REMARKS

Applicants have cancelled claims 1 to 12 without prejudice, and have replaced them with new claims 13 to 31. These new claims add no new matter. Support for the new claims is found in the originally filed claims and throughout the specification.

The Invention

The invention relates to the generation and application of novel retroviral vectors for cell-specific gene transfer. More specifically, the invention relates to retroviral vectors that encompass viral cores derived from the murine leukemia virus (MLV) and envelope proteins derived from human immunodeficiency virus (HIV) or simian immunodeficiency (SIV). These retroviral vectors can be used, for example, to transfer genes into selected cell types, such as CD4-positive mammalian cells. The invention also covers methods of preparing packaging cells, and the packaging cells themselves, that can produce these new retroviral vectors, as well as methods of using the new retroviral vectors.

Priority

Applicants enclose herewith a certified copy of German Patent Application No. 19707971.7 filed on February 27, 1997, along with a copy of a verified translation. Thus, applicants are entitled to the February 27, 1997 filing date.

Claim Objections

Claims 1 to 8 have been rejected for a variety of informalities. New claims 13 to 31 address and overcome these informalities. For example, the term "expression gene" as cited in original claims 4, 5, and 7 has been replaced by the term "expression construct," in the new claims, which is supported, e.g., on page 1, line 22, of the specification as filed. Applicants have taken the Examiner's other suggestions into consideration as well.

35 U.S.C § 112, Second Paragraph

Claims 1-12 have been rejected as being indefinite for failing to particularly point out and distinctly claim the subject matter that applicant regards as the invention.

Applicants submit that new claims 13 to 31 omit or clarify each of the alleged indefinite terms or phrases and thus overcome these rejections.

In particular, applicants make the following comments. To overcome the examiner's objections to claim 1, applicants submit new claim 13, wherein the plural forms of "retroviral vectors, viral cores, virus envelopes and envelope [and transmembrane] proteins" have been changed to singular. Furthermore, the "surface envelope protein" as cited in line 3 of claim 1 has been specified as "full-length surface envelope protein," which is supported on page 3, lines 12-13, of the specification as filed.

The Office Action further indicated on page 4, 2nd paragraph, that it is unclear to which envelope protein the "truncated variant" of claim 1 refers. The "truncated variant" has been further defined by inclusion of the term "transmembrane," which is supported at page 3, lines 12-17, and lines 29-33, of the specification as filed.

The Office Action states that claim 2 is indefinite, because it is not clear to which virus envelopes or what part of the virus envelops are being referred and because the term "derived from" is allegedly unclear. Without agreeing with the Examiner's conclusions about these terms, applicants submit that new claim 15 clarifies these points.

Claim 3 was deemed indefinite for the same reasons as mentioned above for claim 2. Again, applicants submit that the viral envelopes are clearly defined in claim 1, from which new claim 16 depends. The Office Action further indicated that the recitation "which is elongated by the C-terminus" in claim 3 is unclear. To overcome this rejection, applicants have amended the respective passage in new claim 16, which now reads "wherein the truncated variant of the transmembrane envelope protein is modified by fusion to the C-terminus or of any other fragment of the transmembrane envelope protein of a murine leukemia virus (MLV) or of any other retrovirus." This amendment is supported on page 4, lines 2-4, of the specification as filed.

The Office Action also objected to the subject matter of claims 4 and 5 for being allegedly indefinite for various reasons. To overcome these objections, applicants have submitted new claims 17 and 18, which take the Examiner's suggestions into account. New claim 17 is supported, e.g., on page 1, lines 23-24, page 3, lines 19-22, and on page 4, lines 10-15, whereas new claim 18 is supported, e.g., on page 1, lines 23-24, page 3, lines 19-22, and on page 4, lines 17-22, of the specification as filed.

Claim 6 has been replaced with new claim 19 to overcome the lack of insufficient antecedent basis for the "cell line TELCeB6." Claim 7 has been replaced by new claim 20, which deletes the second "or," because the env-expression construct can be either pLßAc/env-Tr712-neo or one of the various pRep env-deletion variants.

The Office Action rejected claim 8 for being incomplete because it allegedly did not make clear which packaging cells are actually obtained. New claim 21 clearly specifies that the term "packaging cells" refers to the "packaging cells" of claim 17.

35 U.S.C § 101

Claims 9-12 have been rejected because according to the Office Action, the claimed recitation of a use, without setting forth any steps involved in the process, results in an improper definition of a process. Applicants have rewritten original claims 9 to 12 as new composition and method claims 22 to 31, which fully meet the requirements of 35 U.S.C. § 101. This rejection should, therefore, not be applied to the new claims.

35 U.S.C § 112, First Paragraph

Claims 6, 7, and 9-12 have been rejected as allegedly containing subject matter that was not described in the specification in such a way as to enable one skilled in the art to make and/or use the invention. Applicants respectfully traverse this rejection in view of the new claims and for the following reasons.

According to the Office Action (at page 8):

> The application discloses cell lines (TELCeB6) and expression plasmids (plβAc/env-Tr712-neo and pMB2) that are encompassed by the definitions for biological material set forth in 37 C.F.R. § 1.801. Because it is apparent that this biological material is essential for practicing the claimed invention, it must be obtainable by a reproducible method set forth in the specification or otherwise be known and readily available to the public as detailed in 37 C.F.R. § 1.801 through 1.809.
>
> The specification does not teach how to reproducibly construct these biological materials from starting materials known and readily available to the public and it is unclear whether this biological material is readily available to the public. Availability of such biological material is deemed necessary to satisfy the enablement provisions of 35 U.S.C. § 112. If this biological material is not

obtainable or available, the requirements of 35 U.S.C. § 112 may be satisfied by a deposit of the biological material.

Applicants submit that the construction of the packaging cell line TelCeB6 cell line is described in Cosset et al., 1995, J. Virol 69(12):7430-7436 (a copy of which is attached as Exhibit 1). The expression plasmid pLßAc/envTr712-neo is disclosed in Wilk et al., 1992, Virology 189:167-177 (already cited by the Examiner in this case), and in Kräusslich et al., 1993, Virology 192:605-617 (Exhibit 2), as already stated on page 4, lines 17-20, of the specification as filed. The starting plasmid pMB2 is described in Baier et al., 1989, J. Virol. 63:5119-5123 (Exhibit 3).

Applicants submit that those of skill in the art can easily prepare the cell lines and plasmids described in the application and carry out the claimed invention by following the teachings in the present specification and the references cited therein. A deposit of these biological materials is therefore not required.

Next, the Office Action states that "[a]lthough claims 9-12 recite non-statutory embodiments not subject to examination, to the extent that the claims are amended to recite pharmaceutical vector compositions or their use in methods for in vivo administration to patients in need thereof, the claims read on gene therapy which are not enabled by the present disclosure" (at page 9). The Office Action also states that the specification "does not provide an adequate written description teaching one of ordinary skill in the art how to make and use the claimed invention to treat any disease using gene therapy (at page 10, last sentence of the Office Action).

Applicants submit that those skilled in the art would know from the prior art how to use the vectors of the present invention for gene therapy. The Office Action recites the factors to be considered in determining enablement as summarized in In re Wands, 858 F. 2d 731, 8 U.S.P.Q.2d 1400 (Fed. Cir., 1998), and then cites various journal articles to support the position that the specification allegedly does not address the problems associated with treating diseases using gene therapy. However, these journal articles do not recite the use of applicants' new retroviral vectors. Applicants respond to the individual points raised in the Office Action as follows.

Nature of the invention and state of the prior art. Applicants acknowledge the Examiner's citation of the Anderson and Verma et al. papers, but note that applicants new retroviral vectors address the so-called "Achilles heel of gene therapy," gene delivery. The disadvantages and problems of gene therapy employing retroviral vectors are discussed in Lodge et al., Gene Therapy 1998, 5: 655-664, "MuLV-based vectors pseudotyped with truncated HIV glycoproteins mediate specific gene transfer in CD4+ peripheral blood lymphocytes" (copy enclosed as Exhibit 4).

Lodge describes certain disadvantages associated with the use of retroviral vectors such as, for example, low transducing efficiency, which is due, in part, to the fact that many retroviral vectors are not targeted to specific cell types (see, e.g., Lodge et al., page 655, left col., lines 10-20, of the section "Introduction").

According to Lodge, the critical point of retroviral gene therapy is that "whenever possible, it will be preferable to use authentic viral envelope glycoproteins exhibiting specificity towards the desired targeted cells" (Lodge et al., page 656, left. col., lines 4-6). Applicants submit that this critical point is embodied by the present application: the retroviral vectors described and claimed in the present application are suitable for selective gene transfer into specific cell types, e.g., CD4+ cells (page 4, lines 27-29, p. 5, lines 11, p. 17, line 25, and p. 18, line 3, of the specification as filed).

Lodge furthermore "confirm[s] and extend[s]" applicants' data in their Schnierle et al. (P.N.A.S., USA, 94:8640-8645, 1997) paper that is also disclosed in the present application, i.e., truncation of the cytoplasmic portion of the Env glycoprotein enables its incorporation into heterologous MLV virions (see, page 656, left col., 3rd para., of Lodge). Lodge also demonstrates that pseudotyped MLV-based vectors specifically infect activated CD4+ peripheral blood lymphocytes (p. 656, left col., 3rd para.).

Lodge concludes, "these results [which correspond to the results obtained in the present application as filed] demonstrate the feasibility of using this approach to specifically transduce genes into the CD4 expressing cell subpopulation for gene therapy purposes" (p. 656, left col., end of 3rd para.). See, also the entire discussion, in which Lodge describes the many benefits of using CD4+ cell-targeted MuLV vectors to deliver HIV replication inhibitors in specific cells in

AIDS patients. Based on these statements, applicants submit that the Lodge paper supports the enablement of the presently claimed invention.

Predictability of the art. According to the Office Action, "the physiological art is recognized as unpredictable (MPEP 2164.03)." Applicants agree that there is some level of unpredictability in gene therapy on an individual cell or patient basis; however, applicants submit that when using the new retroviral vectors of the present application there is an overall predictability of success. Based on the data in the application and as described in the Lodge paper, applicants submit that the use of the new retroviral vectors for gene therapy is enabled, and that successful results are predictable.

Guidance and working examples. The Office Action alleges that, "the specification provides little or no guidance teaching one of ordinary skill in the art how to make or use the retroviral vectors recited for treatment of any disease by gene therapy. No working examples have been provided" (at page 11). Applicants respectfully disagree that "no working examples have been provided," and point to the examples in the application, which describe how to make and use the new retroviral vectors. In particular, Example 4 describes CD4-dependent transduction mediated by the new MLV(SIVagm) vectors (at pages 17-18 of the application as filed). Based on this ability to target CD4+ cells, applicants submit that those of skill in this field would expect that the new retroviral vectors would also work ex vivo and in vivo.

Amount of experimentation necessary. The Office Action states that, "[g]iven the unpredictable and undeveloped state of the art as described above, it would likely require considerable experimentation to appropriately develop the claimed method for treating any disease by gene therapy" (at page 11, emphasis added). Applicants submit that the emphasized portion of the quotation from the Office Action indicates that the Examiner is speculating as the nature of the experimentation required. Although some experimentation may be required, applicants submit that given the teachings in the present specification, and the knowledge available in the art at the time the present application was filed, any experimentation would not be "undue," but would merely be routine. In other words, no new ideas or further invention are required to allow one of skill in this field to practice the claimed invention.

Summarizing, applicants submit that the teachings of the present application, which are confirmed by the publication of Lodge, clearly enable the skilled person to use the new retroviral

vectors in gene therapy. Because these vectors offer the possibility of specifically transducing specific cell, such as CD4+ cells, they circumvent the problems associated with non-targeted vectors, such as amphotropic-MLV-based vectors, which transduce targeted as well as non-targeted cells. Thus, the new vectors overcome the problems discussed in the prior art cited by the Examiner, and this rejection should not be applied to the new claims.

35 U.S.C § 102

Claims 1-8 have been rejected under 35 U.S.C. 102(a) as being clearly anticipated by Schnierle et al., (Proc. Natl. Acad. Sci. USA, 94:8640-8645, 1997).

The Office Action states that applicants cannot rely upon the foreign priority papers to overcome this rejection because a translation of these papers has not been made of record. As noted above, applicants have filed a certified translation of German application No. 197707971.7 along with this response. As a result, this rejection can be withdrawn.

Claims 1-8 have been rejected under 35 U.S.C. 102(f) because the named inventors allegedly did not invent the claimed subject matter. According to the Office Action, Schnierle et al. (1997) discloses the invention of claims 1-8, and notes at the bottom right of p. 8640, that "B. S. S. [Barbara S. Schnierle] and J.S. [Jorn Stitz] contributed equally to this work." Thus, the Office Action concludes that Barbara S. Schnierle is an inventor of the claimed subject matter in the present application.

Applicants agree that the footnote cited in the Office Action states that "B.S.S. and J.S. contributed equally to this work," but respectfully disagree that this statement relates to inventorship of the presently claimed invention. In fact, although Dr. Schnierle clearly worked on several aspects described in the Schnierle et al. paper, she did not work on any subject matter that is claimed in the present application.

To demonstrate that Dr. Schnierle did not work on the claimed invention, applicants submit two additional journal articles from the same laboratory published after the 1997 paper. These two papers further describe the claimed invention, but do not name Dr. Schnierle as a co-author. Thus, Dr. Schnierle contributed only to specific parts of the 1997 paper, but not to the subject matter that is presently claimed. Applicants will now comment on these two papers.

First, Stitz et al., Virology, 2000, 267: 229-236, "MLV-derived retroviral vectors selective for CD4-expressing cells and resistant to neutralization by sera from HIV-infected patients" (copy enclosed as Exhibit 5) focuses on the SIVagm env-pseudotyped MLV vectors, which are part of the subject matter of the present application (see for example claims 13 to 15, and page 3, lines 5 to 7, of the specification as filed). However, as the Examiner can see, Dr. Schnierle is not a co-author of this paper.

Second, Stitz et al., Biogenic Amines, 1998, Vol. 14(5): 407-424, "High-titer retroviral pseudotype vectors for specific targeting of human CD4-positive cells" (Exhibit 6), describes a method of creating packaging cell lines corresponding to the method employed in the present application, i.e., transfection of TELCeB6 cells with plasmid pTr712 (see p. 413, lines 1-3, of the document and page 9, lines 22-23, of the specification as filed) and selection of cell clones (see page 413, line 6, of the document and page 9, line 27 of the specification as filed). Again, Dr. Schnierle is not a co-author of this publication.

Based on these two papers, whose contents correspond to part of the presently claimed invention, it is clear that since Dr. Schnierle is not a co-author of these papers, she did not contribute to the presently claimed invention. Therefore, applicants submit that inventorship of the present application is correct, and that Dr. Schnierle is not a co-inventor of the presently claimed invention.

Next, claims 1-5 and 8 have also been rejected under 35 U.S.C. 102(a) as being allegedly anticipated by Mammano et al. (J. Virol., 71:3341-3345, 1997). Again, the Office Action states that applicants cannot rely upon the foreign priority papers to overcome this rejection because a translation of said papers has not been made of record. As noted above, applicants have filed a certified translation of German application No. 197707971.7 along with this response, so this rejection can be withdrawn.

In addition, claims 4 and 5 have been rejected under 35 U.S.C. 102(b) as being allegedly anticipated by Parolin et al. (J. Virol., 68:3888-3895, 1994). Applicants traverse this rejection for the following reasons.

The Office Action states (at page 14):

> Parolin et al. disclose triple-transfection of a packaging cell (COS-1, expressing SV40 T) antigen) with "expression genes" encoding HIV-1 gag and

> pol (via CMV□P1□envpA plasmid), along with an "an expression construct, comprising a packaging signal (psi) and the genetic information desired to be transferred" (e.g. v653 RSN, for example; see p. 3892, left column) and "an expression gene that contains the genetic information for envelope proteins of HIV or SIV" (pSVIIIenv3-2; refer to cl. 4). It is noted that the claimsdo not specifically recite method steps comprising transfection of "packaging cells" (cl. 4) or "a cell" (cl. 5) comprising MLV "viral cores" with MLV-encoded gag and pol proteins (see also 35 U.S.C. 112 2nd rejection above).

Applicants submit that this rejection should not be applied to the new claims, because Parolin et al. describes packaging cells comprising the HIV-1 gag, pol, vif and tat genes (p. 3889, left col., section "Packaging System"), which are clearly different from the packaging cells in the presently claimed methods for preparing packaging cells. The packaging cells of new claims 17 and 18 encompass "the *gag*-genes and the *pol*-genes of MLV." Therefore, applicants submit that Parolin et al. does not anticipate the subject matter of new claims 17 and 18.

35 U.S.C § 103

Claims 1-6 and 8 have been rejected under 35 U.S.C. 103(a) as being unpatentable over Denesvre et al. (J. Virol., 70:4380-4386, 1996) in view of Salmons et al. (Leukemia, 9 (Suppl.):S53-S60, 1995) and either Wilk et al. (Virology, 189:167-177, 1992) or Zingler et al (J. Virol. 67:2824-2831, 1993). Applicants request that the Examiner not apply this rejection to the new claims for the following reasons.

The Office Action assets (at pages 16-17) that

> It would have been obvious to a personal of ordinary skill in the art at the time the invention was made to incorporate the teachings of heterologous envelope C-terminal truncation and/or substitution mutants and use of the MLV retroviral core cell lines of Denesvre et al. (incl. TELCeBe6) to make the MLV/HIV pseudotypes of Salmons et al. using the truncated HIV envelopes of Wilk et al. or the truncated SIV envelopes of Zingler et al. as well as the accompanying packaging and/or producer cell lines resulting therefrom. One of ordinary skill in the art would have would motivated to utilize the envelope truncation mutants in view of their capacity to promote enhanced heterologous viral particle incorporation, infectivity and fusogenicity, and their established utility for generating MLV pseudotypes, combined with the accompanying predictions for making such pseudotpes as taught by Denesvre et al, and would therefore have predicted a reasonable expectation of success. Thus, the invention

as a whole would have been prima facie obvious to one of ordinary skill in the art at the time the invention was made.

Applicants respectfully disagree for the following reasons. Before the priority date of the present application, there were no reports regarding the generation of HIV/SIV-Env-pseudotyped MLV-based vectors or regarding methods to produce such vectors. Applicants submit that the combination of Denesvre, Salmons, Wilk, and Zingler fails to provide a reasonable expectation of success of preparing the claimed retroviral vectors or the methods to produce them. Applicants will discuss each of the cited references below.

Denesvre describes several experiments investigating the influence of complete, truncated, or chimeric Friend murine leukemia virus (F-MuLV) and human T-cell leukemia virus type 1 (HTLV-1) transmembrane (TM) protein domains on incorporation into virions and on infectivity. As already cited by the Examiner, Denesvre further discloses that "HIV-1 Env containing a long cytoplasmic tail is not incorporated into MuLV particles (p.4385, left col., 3rd para.), and suggests the "simple rule that retroviral cores allow incorporation of heterologous envelopes whose cytoplasmic tails are smaller than that of the original parental envelope" (p.4385, left col., 3rd para).

However, the subject matter of claims 13-19 and 21 of the present invention is directed to HIV/SIV-Env-pseudotyped MLV-based vectors, which are able to efficiently infect CD4+ cells and to packaging cells that produce such vectors. The Office Action admits that Denesvre does "not specifically disclose MLV/HIV or MLV/SIV pseudotype retroviral vectors [or] packaging cell and/or producer cell lines which produce these vectors" (at page 16). Thus, the Office Action looks to Salmons and either Wilk or Zingler to fill the gaps left by Denesvre. However, even this combination of references fails to suggest that it is possible to generate HIV/SIV-Env-pseudotyped MLV-based vectors.

First, the Office Action cites Salmons, which allegedly shows that the HIV-Env protein could be used to target expression of MLV-based retroviruses to CD4+-cells through the formation of a MLV/HIV pseudotype (at page 16 of the Office Action). However, this analysis overlooks the fact that Salmons states that "it appears that a mere co-production of HIV Env in an MLV Gag and Pol producing packaging cell line may not be sufficient to ensure efficient

production of MLV-HIV-pseudotypes (p. S58, left col., 2nd para of section "Discussion", emphasis added). Accordingly, this publication does not predict a reasonable expectation of success in obtaining infectious HIV/SIV-Env-pseudotyped MLV-based vectors. Therefore, applicants submit that the combination of Denesvre and Salmons does not render the subject matter of claims 1-6 and 8 obvious.

Of course, the Office Action also cites either Wilk et al. or Zingler et al. However, neither of these additional references make up for the defects in the primary references. First, Wilk describes the generation of various HIV-1 mutants characterized by the production of env gene products truncated within the cytoplasmic C-terminal tail, which did not lead to loss of in vitro infectivity and cytopathogenicity (page 167, abstract). However, Wilk further states that "the cytoplasmic region in the SIV TM protein must play a role *in vivo* or in growth on certain cell lines" (p.175, right col., end of 2nd para.), and continues that "[t]runcation of the HIV-1 TM protein has not, with one exception, been observed in vivo" (p.175, right col., end of 2nd para., citation omitted). Accordingly, Wilk concludes, "although the cytoplasmic tail of the HIV-1 TM protein is not required for glycoprotein incorporation into virus particles and infectivity in MT-4 cells, it may be required for infectivity in other cell lines and may play a role in vivo" (page 175, right col., last sentence).

In view of these statements, Wilk would not lead a person skilled in the art to generate HIV/SIV-env pseudotyped MLV core particles, wherein the transmembrane envelope protein of HIV/SIV can be a truncated variant. Instead, applicants submit that Wilk would put the person of skill in doubt whether incorporation of a truncated HIV transmembrane envelope protein would lead to infectious virions. Therefore, the combination of Denesvre, Salmons, and Wilk does not render the presently claimed subject matter obvious.

Second, the Office Action cites Zingler, which describes various SIV envelope mutants containing truncated cytoplasmic domains (p. 2824, abstract), which displayed enhanced "envelope density on particles and envelope-mediated cell to cell fusion" (p. 2824, abstract). However, Zingler nowhere describes or suggests the use of truncated SIV envelope proteins to pseudotype MLV-viral cores. As the Examiner is no doubt aware, the prior art, not applicants' application, must contain the requisite motivation to combine all the cited references.

Here, it appears that the Office Action relies on the present application to provide a roadmap to show how to combine the references. Of course, this is improper. Even if the cited prior art is broadly interpreted to suggest that applicants' claimed specific combination might be pieced together based on the separate cited disclosures, a rejection based on this suggestion amounts to no more than an "obvious to try" rejection, which, according to the Federal Circuit, is not the proper standard in an obviousness analysis. Hybritech, Inc. v. Monoclonal Antibodies, Inc., 802 F.2d 1367, 1380 (Fed. Cir. 1986), cert. denied, 480 U.S. 947 (1987) (an invitation to try an experiment is not the proper test for obviousness). The prior art must suggest not only that something may be tried, but also that this try would have a reasonable likelihood of success. In re Dow Chemical Co., 837 F.2d 469, 473 (Fed. Cir. 1988)(emphasis added):

> The consistent criterion for determination of obviousness is whether the prior art would have suggested to one of ordinary skill in the art that this process should be carried out and would have a reasonable likelihood of success, viewed in the light of the prior art. ...(Citations omitted). Both the suggestion and the expectation of success must be found in the prior art, not in the applicant's disclosure.

In the present case, the cited prior art does not suggest applicants' claimed combination or provide a reasonable likelihood of success for that combination. Summarizing, applicants submit that none of the publications cited by in the Office Action, singly or in combination, suggest generating HIV/SIV-Env-pseudotyped MLV-based vectors or the new the methods. The present invention discloses for the first time that a full-length or truncated transmembrane envelope protein of HIV or SIV can be used to pseudotype an MLV-core particle, for use, e.g., to efficiently infect CD4+-cells. Therefore, the subject matter of claims 13-31 is novel and inventive vis-à-vis the cited prior art.

CONCLUSION

Applicants submit that all of the claims are now in condition for allowance, which action is requested. Filed herewith is a Petition for Automatic Extension for three months with the required fee. No excess claims fee is believed due. However, please apply any other charges or

credits to Deposit Account No. 06-1050, referencing attorney docket no. 11692/002001.

Respectfully submitted,

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High-Titer Packaging Cells Producing Recombinant Retroviruses Resistant to Human Serum

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Novel retroviral protein expression constructs were designed to retain minimal retroviral sequences and to express dominant selectable markers by reinitiation of translation after expression of the viral genes. HT1080 cells were selected as producer cells for their ability to release high-titer viruses that are resistant to inactivation by human serum. Two HT1080-based packaging cell lines which produce Moloney murine leukemia virus cores with envelope glycoproteins of either amphotropic murine leukemia virus (FLYA13 line) or cat endogenous virus RD114 (FLYRD18 line) are described. Direct comparison with previous retroviral packaging systems indicated that 100-fold-higher titers of helper-free recombinant viruses were released by the FLYA13 and FLYRD18 lines.

Some experimental and clinical gene transfer protocols require the design of gene transfer vectors suitable for in vivo gene delivery (19). Retroviral vectors are attractive candidates for such applications because they can provide stable gene transfer and expression (28) and because packaging cells which produce non-replication-competent viruses have been designed (18). However, currently available recombinant retroviruses suffer from a number of drawbacks. First, such vectors are rapidly inactivated by human serum, which makes them inappropriate for many clinical in situ gene transfer protocols. Second, the current split packaging function systems provide limited titers of infectious retroviral vectors (10^5 to 10^7 infectious units [i.u.] per ml). In these cells, the two helper genomes have been introduced by cotransfection with plasmids encoding selectable markers (7, 17, 20). Thus, no direct selection is applied to the packaging genome itself, and helper functions can be lost during passage of the cells in culture (6). Third, while the retroviral vectors prepared from split packaging function cell lines are usually not contaminated by replication-competent retroviruses (RCRs), other types of recombinant retroviruses have been shown to arise spontaneously from such cells (4, 9, 22, 31).

Here, we report the construction of new packaging cell lines designed to overcome these constraints. First, we have previously shown that inactivation by complement in human serum is controlled by the cell line used to produce the virions and by viral envelope determinants (37). We now describe packaging cells that will produce complement-resistant virus using human HT1080 cells and cat virus RD114 envelope. Second, we established direct selection for expression of packaging functions by expressing selectable marker genes after viral genes by reinitiation of translation. Finally, the generation of RCRs involves multistep recombinations between vector plasmids and *gag-pol* and *env* genomes provided either by the packaging plasmids (4, 9, 22) or by endogenous retroviral sequences (26, 40). The possibility of generation of such RCRs was therefore reduced by decreasing the viral sequences in the helper constructs. The new packaging cell lines were able to transduce helper-free, human complement-resistant retroviral vectors at titers consistently higher than 10^7 i.u./ml.

MATERIALS AND METHODS

Cell lines and viruses. Cell lines A204 (ATCC HTB 82), HeLa (ATCC CCL2), HT1080 (ATCC CCL121), MRC5 (ATCC CCL171), T24 (ATCC HTB 4), Vero (ATCC CCL81), and D17 (ATCC CCL183) were purchased from the American Type Culture Collection. HOS, TE671, and Mv-1-Lu cells and their clones harboring the MFGnlslacZ retroviral vector have been described previously (37). All these cell lines were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) supplemented with 10% fetal calf serum.

EB8 (1), psiCRE, psiCRELLZ, and psiCRIP (7) cells were kindly provided by O. Danos and J. M. Heard. GP+EAM12 (17) cells were kindly provided by A. Bank. These cell lines and also NIH 3T3 murine fibroblasts were grown in DMEM supplemented with 10% newborn calf serum.

Mv-1-Lu, TE671, and HT1080 cells were transfected by the calcium phosphate precipitation method (29) as described elsewhere (1). CeB-transfected Mv-1-Lu, TE671, and HT1080 cells were selected with 3, 6 to 8, and 4 μg of blasticidin S (ICN) per ml, respectively, and blasticidin-resistant colonies were isolated 2 to 3 weeks later. Cells transfected with the various *env* expression plasmids were selected with phleomycin (CAYLA): 50 μg/ml for ALF-transfected cells and 10 μg/ml for AXF-, AF-, and RDF-transfected cells. Phleomycin-resistant colonies were isolated 2 to 3 weeks later.

The production of LacZ pseudotype viruses from replication-competent viruses, amphotropic murine leukemia virus (MLV-A) strain 1504, and cat endogenous virus RD114 was carried out as described previously (37).

Plasmids. The *env* gene of pCRIP (kindly provided by O. Danos and J. M. Heard) (7) was excised by *Hpa*I-*Cla*I digestion. A 500-bp PCR-generated DNA fragment was obtained from pSV2-bsr (kindly provided by F. Hanaoka) (12) as the template and a pair of oligonucleotides (5′-CGGAATTCGGATCCGAGCT CGGCCCAGCCGGCCACCATGAAAACATTTAACATTTCTC at the 5′ end and 5′-GATCCATCGATAAGCTTGGTGGTAAAACTTTT at the 3′ end) with *Sfi*I and *Cla*I sites, respectively. This fragment was inserted into the *Hpa*I and *Cla*I sites of pCRIP by coligation with an 85-bp *Hpa*I-*Sfi*I DNA fragment isolated from pOXEnv (kindly provided by S. J. Russell) (27), which provides the end of the Moloney murine leukemia virus (MoMLV) *pol* gene. The resulting plasmid, named CeB (Fig. 1), could express the MoMLV *gag-pol* gene as well as the *bsr* selectable marker, conferring resistance to blasticidin S, both driven by the MoMLV 5′ long terminal repeat (LTR) promoter.

A series of *env* expression plasmids were generated by using the 4070A

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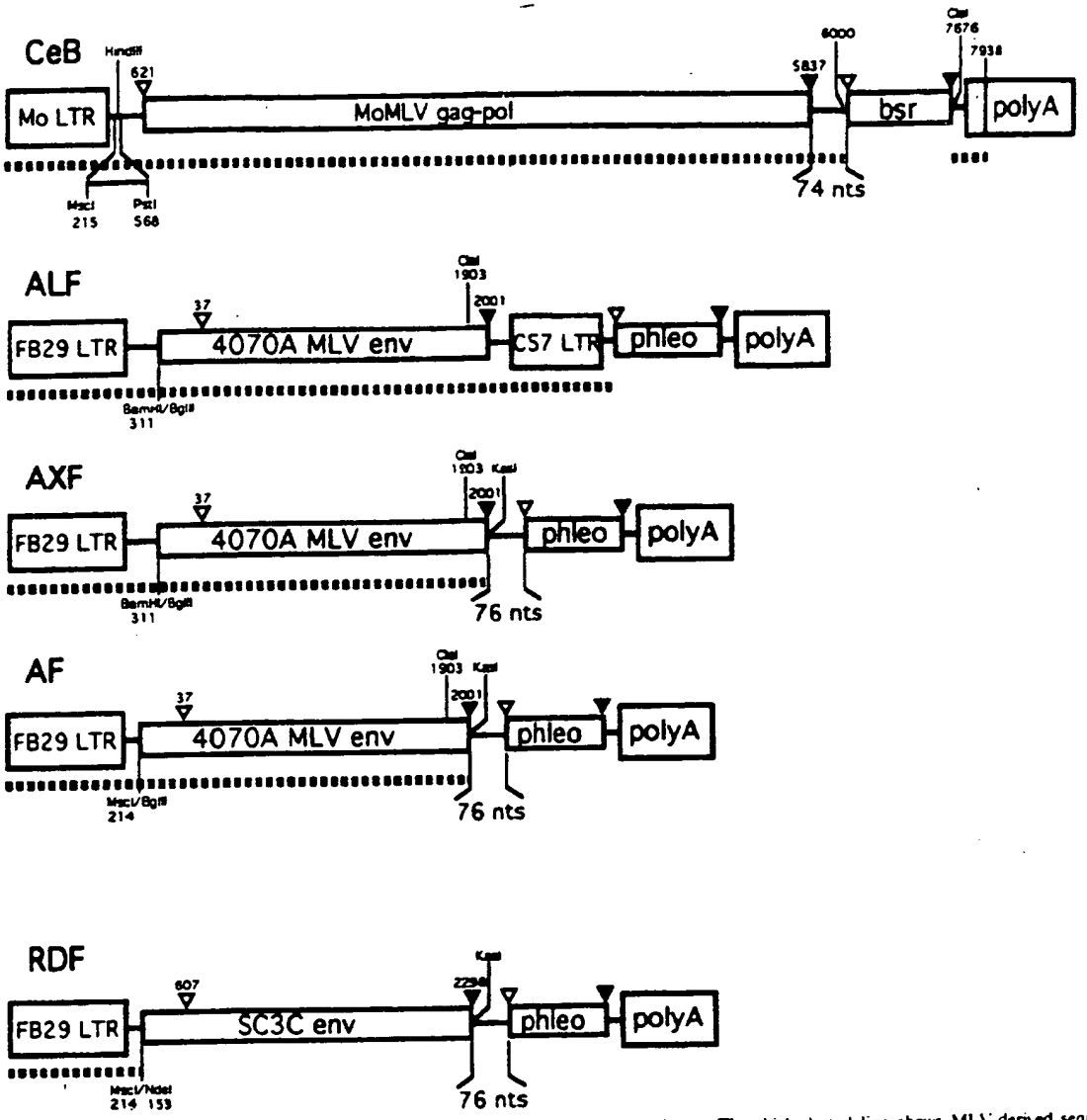

FIG. 1. Schematic diagrams of constructs. Initiation (▽) and termination (▼) codons are shown. The thick dotted line shows MLV-derived sequences. The nucleotide positions of MLV-derived sequences are shown according to Shinnick et al. (33) for CeB (from nt 1 to nt 6000, with deletion of the packaging signal from *Bal*I [nt 215] to *Pst*I [nt 568] and with some further MoMLV sequences from nt 7676 to nt 7938), Perryman et al. (23) (from nt 1 to nt 311 or 214), and Ott et al. (24) (from *Bgl*II to nt 2001) for ALF, AXF, and AF and Perryman et al. (23) (from nt 1 to nt 214) for RDF. Numbering of the RD114 sequence for RDF (from nt 153 to nt 2298) is as reported (EMBL accession number X87829).

MLV-A *env* gene (23) and the FB29 Friend MLV promoter (24). In ALF (Fig. 1), a *Bgl*II-*Cla*I fragment containing the *env* gene was cloned into the *Bam*HI and *Cla*I sites of plasmid FB3LPh (1a), which also contained the C57 Fnend MLV LTR driving the expression of the phleomycin resistance (*phleo*) selection marker (8). A 136-bp *env* fragment was generated by PCR with plasmid FB3 (10) as the template and a pair of oligonucleotides, 5'-GCTCTTCGGACCCTGC ATTC at the 5' end (before the *Cla*I site) and 5'-TAGCATGGCGCCCTATGG CTCGTACTCTATAGGC at the 3' end, providing a *Kas*I restriction site immediately after the *env* stop codon. This PCR fragment was digested with *Cla*I and *Kas*I. A DNA fragment containing the FB29 LTR and the MLV-A *env* gene was obtained by *Nde*I-*Cla*I digestion of ALF. The fragments were coligated in *Nde*I-*Kas*I-digested pUT626 (kindly provided by Daniel Drocourt, CAYLA). In the resulting plasmid, named AXF (Fig. 1), the *phleo* selectable marker was expressed from the same mRNA as the *env* gene. A *Bgl*II restriction site was created after the *Msc*I site at position 214 in the FB29 leader by using a commercial linker (Biolabs). An *Nde*I-*Bgl*II fragment containing the FB29 LTR was coinserted with the *Bgl*II-*Cla*I *env* fragment into *Nde*I-*Cla*I-digested AXF plasmid DNA, resulting in plasmid AF (Fig. 1). AF has a 100-bp-larger deletion in the leader region than AXF.

The RD114 *env* gene was first subcloned in plasmid Bluescript KS- (Stratagene) as a 3-kb *Hin*dIII insert isolated from SC3C, an RD114 infectious DNA clone (kindly provided by S. O'Brien) (25). This *env* gene was sequenced (EMBL accession number X87829). The 5' noncoding sequence upstream of an *Nde*I site was deleted by *Eco*RI and *Nde*I digestion followed by filling-in with Klenow enzyme and self-ligation. From this plasmid, two DNA fragments were obtained: a *Bam*HI-*Nco*I 2.5-kb fragment and a 63-bp PCR-generated DNA fragment (using 5'-CGCCTCATGGCCTTCATTAA at the 5' end [before the *Not*I site] and 5'-TAGCATGGCGCCTCAATCCTGAGCTTCTTCC at the 3' end), providing a *Kas*I restriction site just after the RD114 *env* gene stop codon. The PCR fragment was digested with *Nco*I and *Kas*I. Both fragments were coinserted between the *Bgl*II and *Kas*I sites of AF, and the resulting plasmid was named RDF (Fig. 1).

Plasmid pCRIPAMgag⁻ (7) (kindly provided by O. Danos and J. M. Heard) was used for transfection.

Infection assays. Target cells were seeded in 24-multiwell plates (4×10^4 cells per well) and incubated overnight. Infections were then carried out at 37°C by plating 1 ml of dilutions of viral supernatants in the presence of Polybrene (4 μg/ml; Sigma) on target cells. After 3 h, virus-containing medium was replaced by fresh medium, and infected cells were incubated for 2 days before X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining, performed as previously described (36, 37). Viral titers were determined by counting LacZ-positive colonies as previously described (5). The stability of LacZ pseudotype viruses

TABLE 1. Titer and stability of LacZ pseudotypes[a]

Producer cells	LacZ(MLV-A)		LacZ(RD114)	
	Titer (i.u. ml)	Stability (% of control)	Titer (i.u. ml)	Stability (% of control)
A204	650	<3	1,200	105
HeLa	9	ND[b]	2,000	115
HOS	4,500	6	23,000	86
HT1080	2,000,000	26	400,000	129
MRC-5	450	10	1,000	ND
T24	350	ND	1,200	ND
TE671	15,000	2	90,000	38
Vero	260	ND	90	ND
D17	900	<1	200,000	1
Mv-1-Lu	80,000	1	200,000	120

[a] Titer was determined on TE671 cells. Stability is expressed as the infectivity of human serum-treated viruses as a percentage of that of fetal calf serum-treated viruses.
[b] ND, not done.

in fresh human serum was examined by titrating surviving virus after incubation in a 1:1 mixture of virus harvest in serum-free medium and fresh human serum for 1 h at 37°C as described before (37).

RT assays. The reverse transcriptase (RT) assays were performed either as described previously (37) or with an RT assay kit (Boehringer Mannheim) following the manufacturer's instructions but using $MnCl_2$ (2 mM) instead of $MgCl_2$.

Nucleotide sequence accession number. The EMBL accession number for the sequence of the RD114 *env* gene is X87829.

RESULTS

Screening of producer cell lines. We have shown that viral particles generated with RD114 envelopes are more stable in human serum than virions with MLV-A envelopes and that the producer cell line also controls sensitivity (37). We have also found that viruses produced by certain nonprimate cells are sensitive to human serum because they bear Gal(α1-3)galactosyl sugar on their envelope, which activates complement via "natural" antibodies against this sugar epitope present in human serum (38). We therefore screened a panel of cell lines, mostly of primate origin, for their ability to produce high-titer viruses and for the sensitivity of these virions to human serum. To do this, cells were infected at high multiplicity with LacZ pseudotype viruses of either MLV-A or RD114, and cells producing helper-positive LacZ pseudotype viruses were established. Human HT1080 and TE671 and mink Mv-1-Lu cells were found to release high-titer LacZ(RD114) and LacZ (MLV-A) viruses. The LacZ(MLV-A) pseudotype viruses produced by HT1080 cells were more resistant to human serum than those produced by other cells. The titer of these viruses was only fourfold lower following a 1-h incubation with human serum than in a control incubation with fetal calf serum (Table 1). The LacZ(RD114) pseudotype viruses produced by human cells and mink Mv-1-Lu cells were in general stable in human serum (Table 1). These results suggested that HT1080, TE671, and Mv-1-Lu cells provided the best combination of high LacZ virus titers and resistance to human serum, and they were therefore used for the generation of retroviral packaging cells.

Construction of an improved *gag-pol* expression vector. An MoMLV *gag-pol* expression plasmid, CeB (Fig. 1), was derived from pCRIP (7). Approximately 2 kb of *env* sequence were removed from pCRIP, and the *bsr* selectable marker, conferring resistance to blasticidin S (12), was inserted 74 nucleotides (nt) downstream of the *gag-pol* gene. This 74-nt interval had no ATG triplets and was thought to provide an optimal distance between the stop codon of the *pol* reading frame and the start codon of the *bsr* gene to allow reinitiation of translation (15). Therefore, *bsr* could only be expressed by reinitiation of translation after the upstream *gag-pol* gene had been expressed. Consequently, after transfection of CeB into Mv-1-Lu/MFGnlsLacZ (ML), TE671/MFGnlsLacZ (TEL), or HT1080 cells, blasticidin S-resistant bulk populations and most cell clones expressed high levels of Gag-Pol proteins, as assessed from the RT activity found in cell supernatants (Table 2). Considerably higher RT activities were found in bulk populations of CeB-transfected ML cells than in bulk populations of ML cells stably transfected with the parental pCRIP construct. Similarly, the RT activities of two packaging cell lines generated by using the pCRIPenv$^-$ construct, psiCRE cells (7) and EB8 cells (1), were less than that of CeB-transfected clones (Table 2). Indeed, RT activity in CeB-transfected cell supernatants was higher than that of cells chronically infected by replication-competent MLV-A (Table 2).

To rescue infectious LacZ viruses, MLCeB and TELCeB clones were transfected with ALF DNA, a plasmid designed to express the MLV-A *env* gene (Fig. 1). Bulk populations of stable ALF transfectants were isolated, and supernatants were titrated by using TE671 cells as targets. Titers of LacZ viruses were higher than in either MLV-A-infected ML or TEL cells or ALF-transfected EB8 cells (Table 2). These data suggested that CeB was an extremely efficient MLV *gag-pol* expression vector in mink Mv-1-Lu and TE671 cells. We therefore used CeB to derive packaging cells by transfection of HT1080 cells. Forty-one of 49 blasticidin S-resistant colonies had detectable levels of RT: 9 had RT activity higher than that of control MLV-A-infected HT1080 cells (data not shown). Expression of the Gag precursor was confirmed in cell lysates and supernatants

TABLE 2. Secreted RT expression

Cells[a]	RT activity[b]	LacZ virus titer (i.u./ml)
ML/MLV-A	1	8×10^4
MLSvB	0.1	<1
MLCRIP (bulk)	0.15	ND[d]
MLCeB (bulk)	1.7	ND
MLCeB1	4.2	1×10^6
MLCeB4	1.6	1×10^6
TEL/MLV-A	3.6	2×10^6
TELCeB6	5.2	4×10^7
HT1080/MLV-A	1.1	1×10^6
HTCeB6	1.9	1×10^6
HTCeB18	2.7	2×10^6
HTCeB22 (FLY)	6.9	5×10^6
HTCeB48	5.5	3×10^6
EB8	0.22	1×10^4
psiCRE-LLZ	1.2	1×10^5[e]

[a] ML, Mv-1-Lu cells harboring a MFGnlslacZ provirus; TEL, TE671 cells harboring a MFGnlslacZ provirus; MLV-A, cells chronically infected with MLV-A strain 1504; MLSvB, ML cells transfected with plasmid pSV2bsr alone; MLCRIP, ML cells cotransfected with pCRIP and pSV2bsr.
[b] Average of ratios of RT activity relative to that released by ML/MLV-A cells of at least two independent experiments is shown. The standard errors did not exceed 20% of the values.
[c] Titer determined on TE671 cells (except as noted) after polyclonal transfection of a plasmid which expresses MLV-A *env* in MLCeB clones, TELCeB clones, HTCeB clones, and EB8 cells.
[d] ND, not done.
[e] Titer determined on NIH 3T3 cells.

TABLE 3. Titers following *env* construct transfection[a]

Producer cells	*env* source	Titer (i.u. ml)
psiCRIP lacZ 5[b]	pCRIPAMgag$^-$	6×10^4
GP+EAM12 lacZ 25[b]	envAM	3×10^5
TELCeB6[c]	ALF	5×10^7
	AXF	2×10^7
	AF	2×10^7
TELCeB6	AF 1	3×10^7
	AF 4	2×10^7
	AF 6	1×10^7
	AF 7	5×10^7
	AF 8	1×10^7
	RDF 2	1×10^6
	RDF 4	3×10^5
	RDF 7	1×10^7
	RDF 8	2×10^6
FLY[d]	AF 1	1×10^4
	AF 4	1.5×10^6
	AF 5	1×10^6
	AF 7	1×10^6
	AF 13	7×10^6
	AF 14	4×10^6
	AF 15	1×10^6
	AF 16	5×10^6
	AF 17	6×10^6
FLYA4 lacZ 3[b]	AF 4	2×10^7
FLY[d]	RDF 1	2.5×10^6
	RDF 2	1×10^7
	RDF 6	5×10^6
	RDF 10	2×10^6
	RDF 11	3×10^6
	RDF 13	1×10^6
	RDF 17	5×10^6
	RDF 18	3×10^7
	RDF 19	6×10^6

[a] Average titers of at least three independent experiments are shown. The standard errors did not exceed 30% of the titer values. Titers were determined on TE671 cells (LacZ virus i.u. per milliliter).
[b] Best MFGnlslacZ producer clones.
[c] Bulk populations of *env* transfectants in TELCeB6 cells.
[d] Titration after bulk infection with helper-free MFGnlslacZ.

tants of these nine HTCeB clones by immunoblotting with antibodies against p30-CA (data not shown). The four clones with the highest expression of Gag proteins (clones 6, 18, 22, and 48) were infected at high multiplicity with helper-free LacZ pseudotypes bearing MLV-A envelopes [MFGnlslacZ (A)] produced by TELCeB6/ALF (Table 3) and then transfected with ALF. Supernatants of bulk, phleomycin-resistant transfectants were assessed for RT activity and LacZ virus titer (Table 2). Clone HTCeB22, named FLY, was found to be the best Gag-Pol producer clone and was used to introduce *env* expression vectors for the generation of packaging cell lines.

Construction of *env* expression vectors. A series of MLV-A *env* expression plasmids were then generated (Fig. 1). In ALF, the *env* gene was inserted between two Friend-MLV LTRs, its expression driven by the FB29 MLV LTR (24). Most of the packaging signal located in the leader region was deleted. This plasmid also expressed the *phleo* selectable marker (8) driven by the 3′ LTR. AXF and AF were then designed following the same strategy used for CeB. These two vectors differed only by the extent of deletion of the packaging signal, AF having virtually no leader sequence. Compared with the pCRIPAMgag$^-$ and pCRIPgag-2 *env* plasmids expressed in psiCRIP and psiCRE packaging cells (7), about 5 kb of *gag-pol* sequence was removed. In addition, the 258-bp retroviral sequence containing the end of the *env* gene and the beginning of U3 found in pCRIPAMgag$^-$ and pCRIPgag$^-$2 was also removed. For both the AXF and AF plasmids, the *phleo* selectable marker was inserted downstream of the *env* gene by positioning a 76-nt linker with no ATG codons between the two open reading frames. *phleo* could therefore only be expressed by reinitiation of translation by the same ribosomal unit that had expressed the upstream *env* open reading frame. AF was also used to generate RDF, an RD114 envelope expression plasmid (Fig. 1).

After transfection of the *env* plasmids into TELCeB6 cells (Table 2), bulk populations of phleomycin-resistant colonies were isolated, and their production of LacZ virus was measured (Table 3). ALF gave a titer of 5×10^7 LacZ virus i.u. ml, while the titers with AXF and AF were 2×10^7 LacZ virus i.u./ml (Table 3). Titers of 5×10^7 and 10^7 LacZ virus i.u. ml could be obtained with some AF cell clones and RDF clones, respectively.

As AF has minimal virus-derived sequences and was shown to be the safest construct (see below and Table 4), it and RDF were used to generate packaging lines from FLY cells (clone HTCeB22 [Table 2]). Envelope expression of these clones was assayed by interference to challenge with MFGnlslacZ(A) and MFGnlslacZ(RD) pseudotype viruses produced by TELCeB6 AF-7 and TELCeB6/RDF-7, respectively (Table 3). The cell lines showing the most interference were cross-infected at high multiplicity with these pseudotype viruses to provide MFGnls lacZ proviruses, and supernatants were then titrated on TE671 cells (Table 3). FLY-AF-13 (FLYA13 packaging line) and FLY-RDF-18 (FLYRD18 packaging line) gave the highest productions of LacZ viruses, about 10^7 LacZ virus i.u./ml. The best MFGnlslacZ producer clones, derived from either psiCRIP cells (7) or GP+EAM12 cells (17), gave approximately 50-fold-lower titers (Table 3). The LacZ virus titers of the FLY-derived lines shown in Table 3 are lower than those of the best TELCeB6-derived lines after transfection of either AF or RDF (Table 3). However, it should be noted that the LacZ provirus expressed in TELCeB6 cells was obtained after clonal selection but was introduced polyclonally in FLY-derived *env*-transfected cell clones. When FLY-AF-4 cells (FLYA4 packaging line), infected with helper-free MFGnlslacZ(RD), were cloned by limiting dilution, the best clones (e.g., FLYA4lacZ3) were found to produce 20 times more infectious viruses than the bulk population, reaching the range of titers obtained with the best TELCeB6-AF clones (Table 3).

Assays for transfer of *gag-pol* and *env* functions. To assay for replication-competent viruses, supernatants were used to infect TEL cells (a clone of TE671 cells harboring an MFGnls lacZ provirus). Infected cells were passaged for 6 days or longer, and their supernatants were used for infection of fresh TE671 cells. No transmission of LacZ viruses could be detected (Table 4), demonstrating that the supernatants of pCRIPAMgag$^-$-, ALF-, AXF-, or AF-transfected TELCeB6 cells were helper-free. A similar absence of replication-competent recombinant retroviruses was demonstrated by using the supernatant from a clone of psiCRIP-MFGnlslacZ cells or two clones of FLYA-MFGnlslacZ cells (Table 4).

There have been reports that helper-free retroviral vector stocks may nevertheless contain recombinant retroviruses (replication incompetent) carrying either *gag-pol* or *env* genes (4, 9, 16). To assay for such recombinant retroviruses, we attempted to mobilize an MFGnlslacZ provirus from two indicator cell lines, which could cross-complement potential re-

TABLE 4. Transfer of packaging function

Producer cells	Indicator cells	Input virus[a] (i.u./ml)	No. of 4 expts giving indicated result[b]		
			++	+	-
RCRs					
psiCRIP lacZ 5	TEL	2×10^4	0	0	4
TELCeB6-pCRIPAMgag	TEL	5×10^6	0	0	4
TELCeB6-AXF	TEL	5×10^6	0	0	4
TELCeB6-AF	TEL	5×10^6	0	0	4
FLYA4 lacZ 3	TEL	1×10^7	0	0	4
FLYA4 lacZ 7	TEL	1×10^7	0	0	4
GPRs					
TELCeB6-AF 7	TELMOXF	2×10^7	0	1	3
		2×10^6	0	2	2
		2×10^5	0	2	2
		2×10^4	0	0	4
ERs					
TELCeB6-pCRIPAMgag	TELCeB6	5×10^6	2	1	1
		5×10^5	1	1	2
		5×10^4	0	2	2
TELCeB6-AXF	TELCeB6	5×10^6	0	2	2
		5×10^5	0	1	3
		5×10^4	0	1	3
TELCeB6-AF	TELCeB6	5×10^6	0	1	3
		5×10^5	1	3	0
		5×10^4	0	0	4

[a] Number of LacZ virus i.u. used to infect indicator cells.

[b] Results from four experiments in which indicator cells were exposed to 1 ml of virus in each experiment. The ranges of LacZ virus titers rescued from infected indicator cells are shown for each virus input: ++, >100 LacZ virus i.u./ml; +, 1 to 100 LacZ virus i.u./ml; -, <1 LacZ virus i.u./ml. Titers were determined on TE671 cells for RCRs and ERs and on NIH 3T3 cells for GPRs.

combinant viruses. The TELCeB6 line (Table 2), expressing Gag-Pol proteins, was used as the indicator cell line to test for the presence of *env* recombinant (ER) viruses. The TELMOXF indicator line, expressing MoMLV *env* glycoproteins (obtained by transfection of MOXF, a plasmid expressing the MoMLV *env* gene using the AXF backbone, into TEL cells), was used to detect the presence of *gag-pol* recombinant (GPR) retroviruses. After passaging for 4 to 8 days, the supernatants of the infected indicator cells were used to infect either human TE671 cells or murine NIH 3T3 cells.

TELCeB6 cells transfected with the various *env*-expressing constructs, pCRIPAMgag$^-$, AXF, and AF, were compared. Although the supernatants of TELCeB6-AF cells were devoid of replication-competent retroviruses, they were found sporadically to transfer *gag-pol* genomes (Table 4). No GPR viruses could be detected when fewer than 2×10^6 virions were used to infect the indicator cells. Similarly, TELCeB6 indicator cells infected with various helper-free viruses were shown to sporadically release LacZ virions (Table 4). The number depended both on the *env* expression vector used and on the virus input quantity. Compared with LacZ viruses generated by using the pCRIPAMgag$^-$ plasmid, the frequency of detection of the ER viruses was lower for supernatants generated by using AXF and AF constructs (Table 4). For the AF construct when fewer than 5×10^5 MFGnlslacZ(A) helper-free virions were used to infect the indicator cells, no ER retroviruses could be detected. From these experiments, it could be estimated that a supernatant produced from TELCeB6-AF cells, containing 10^7 i.u. of MFGnlslacZ retroviral vector, contained less than one replication-competent virus and about 100 GPR and 100 ER retroviruses.

Complement resistance and helper-free status of LacZ vectors produced by FLYA13 and FLYRD18 cells. In order to confirm the resistance to complement and the absence of replication-competent virus in our best packaging lines, MFGnlslacZ(A) and MFGnlslacZ(RD), harvested from FLYA13 and FLYRD18, respectively, after polyclonal transduction of MFGnlslacZ (Table 3) were tested for stability in fresh human serum and generation of replication-competent virus. The titers of MFGnlslacZ(RD) from FLYRD18 after 1 h of incubation with three independent samples of fresh human serum were 80 to 120% of those in control incubations without serum, while the titers of MFGnlslacZ(A) from FLYA13 were 50 to 90% of control titers (data not shown). No replication-competent virus was detected in the assay described above (Table 4) when 10^7 i.u. each of MFGnlslacZ(A) and MFGnlslacZ(RD) were tested.

DISCUSSION

We describe novel retroviral packaging cells, called FLY cells, designed for in vivo gene delivery. The retroviral vectors prepared from these cells are not inactivated by human serum and can be obtained at much higher titers, unlike previous vectors.

We found considerable variations between the various cell lines screened for their ability to release type C retroviruses. In addition, few cell lines were able to produce retroviruses that were completely resistant to human serum. By these two criteria, human fibrosarcoma HT1080 cells were selected for the construction of packaging cells. Other studies have shown the importance of endogenous retrovirus expression in the generation of recombinant retroviruses from retroviral packaging lines (26, 40). The copackaging of an endogenous genome and a vector can lead to the emergence of recombinant retroviruses (40). Recombination involves template switching during reverse transcription of such hybrid retroviruses (11), and homologies between the two genomes considerably enhance the frequency of RT jumps (41). Therefore, an ideal packaging cell line should not express endogenous MLV-like (or type C retrovirus-like) retroviral genomes which can be packaged by type C Gag proteins (30, 39). In recent studies (23a), we could not detect expression of type C retroviruses in HT1080 cells by PCR analysis with generic primers (32), suggesting that HT1080-derived FLY packaging cells may be safer in this respect than those generated from NIH 3T3 cells, which are known to express and package sequences related to type C retroviruses (30).

To generate the FLY packaging cell lines, HT1080 cells were transfected with *gag-pol* and *env* expression plasmids designed to optimize viral protein expression. Direct selection for viral gene expression was achieved by expression of a selectable marker gene by reinitiation of translation of the mRNA expressing the viral proteins. This strategy resulted in packaging cell lines capable of producing extremely high-titer viruses. Furthermore, selection for packaging functions can be maintained in these cells. We also eliminated many unnecessary viral sequences from the packaging constructs to reduce the risk of helper virus generation. The final packaging cells did not produce helper virus, in that no RCR could be detected per 10^7 vector particles, although it must be noted that only a small volume of viral supernatant was assayed. However, it is of concern that recombinant retroviruses expressing either MLV Gag-Pol or Env proteins could be detected. Our data demonstrate that retroviral vector stocks are contaminated

with such recombinant viruses at about 1 per 10^5 vector particles. The new FLY packaging cells are safer than, for example, psiCRIP cells, at least for the generation of ER retroviruses in a short-term assay (Table 4), probably because fewer retroviral sequences overlapping the vector were present in our *env* expression plasmid. Few reports have addressed the question of the characterization of recombinant retroviruses (4, 9). It is possible that such viruses could not be detected in previous packaging cell lines because of low overall titers. Recombinant retroviruses are defective in normal cell culture conditions but are likely to evolve to replication-competent viruses if they are allowed to replicate in cells complementing their expression, such as cocultivated packaging cells (3, 4). They may modify other viruses, such as human immunodeficiency virus or endogenous viruses, by phenotypic and genetic mixing in gene therapy recipients. It will be important for the future development of retroviral packaging systems to eradicate recombinant retroviruses, perhaps by removal of viral LTRs from the packaging construct.

Consistent with our previous studies (37), LacZ(RD114) pseudotypes produced from HT1080 cells were more resistant to human complement than LacZ(MLV-A) pseudotypes. We therefore decided to use the RD114 *env* gene to generate recombinant virions with MoMLV cores. We have determined the sequence of the RD114 *env* gene and found it to be very close to that of baboon endogenous virus, a type C retrovirus (2, 13) with an envelope gene displaying similarities to the external part of type D simian retroviruses. RD114 uses the simian retrovirus receptor on human cells (34, 35), making the FLY packaging cells with the RD114 envelope capable of generating virions with different tropisms. The retroviral vectors prepared so far for human gene therapy have used either MLV-A or gibbon ape leukemia virus envelopes, which display many similarities (1) and which use two related cell surface receptors for infection (21). Differences in tissue-specific expression of the MLV-A and the gibbon ape leukemia virus receptors have been reported (14), and it will now be interesting to see whether particular cell types can be recognized by RD114 Env-coated retroviral vectors.

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ADDENDUM IN PROOF

The FLYA13 and FLYRD18 cell lines will soon be available for research purposes from the European Collection of Cell Cultures under ECACC accession numbers 95091901 and 95091902, respectively.

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Analysis of Protein Expression and Virus-like Particle Formation in Mammalian Cell Lines Stably Expressing HIV-1 *gag* and *env* Gene Products with or without Active HIV Proteinase

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Cell lines stably releasing noninfectious virus-like particles containing wild type or mutant gene products represent useful tools for a biochemical, immunological, and structural analysis of virus assembly. Human immunodeficiency virus (HIV) type 1 *gag* and *env* gene products were transiently and stably expressed in mammalian cells and the formation of virus-like particles incorporating viral glycoproteins was analyzed. Transient cotransfection of plasmids directing the synthesis of *gag* and *env* gene products yielded efficient release of particles but specific incorporation of HIV glycoproteins was not detected. A stable cell line expressing wild type HIV-1 glycoproteins was generated and transient transfection of this cell line with *gag*-encoding constructs led to the release of virus-like particles incorporating HIV surface and transmembrane glycoproteins. Attempts to establish stable cell lines expressing wild type HIV *gag* and *pol* genes were unsuccessful and only highly unstable lines primarily expressing uncleaved precursor polyproteins were obtained. This result appears to be caused by the cytotoxic effects of the viral proteinase since stable lines were readily selected after transfection of constructs either encoding an inactive mutant of the proteinase or a mutated frameshift signal which prevented expression of the *pol* reading frame. Stable coexpression of uncleaved Gag polyprotein and wild type *env* gene products yielded efficient release of immature virus-like particles incorporating HIV glycoproteins. Electron micrographs revealed lentiviral budding structures with the typical surface projections of viral glycoprotein oligomers.

INTRODUCTION

In the late stages of the human immunodeficiency virus (HIV) replication cycle, the immature viral ribonucleoprotein core is assembled at the plasma membrane and subsequently released from the host cell via a budding process involving its envelopment by a lipid bilayer that also contains the viral glycoproteins (reviewed in Gelderblom, 1991). Condensation of the immature spherical core shell to the typical lentiviral cone-shaped core is necessary for the released virion to become infectious and depends on proteolytic processing of the precursor proteins by the viral proteinase (PR, nomenclature, Leis *et al.*, 1988; reviewed in Kräusslich and Wimmer, 1988). Morphogenesis of infectious virus therefore requires the morphopoetic function of the structural components of the viral core (encoded by the *gag* gene) as well as the incorporation of functional replication enzymes (PR; reverse transcriptase, RT; integrase, IN; derived from the *pol* gene) and the viral glycoproteins, gp120 (SU) and gp41 (TM). In contrast to the requirement for *pol* and *env* gene products for viral infectivity, formation of noninfectious, morphologically immature virus-like particles appears to depend solely on the expression of the Gag polyprotein and does not require proteolytic processing or the interaction with glycoproteins. Accordingly, several groups have recently reported HIV polyprotein synthesis and the production of recombinant virus-like particles after infection of different host cell lines with recombinant vaccinia- and baculoviruses expressing the Gag proteins alone or in combination with other HIV gene products (Karacostas *et al.*, 1989; Haffar *et al.*, 1990; Shioda and Shibuta, 1990; Hu *et al.*, 1990; Gheysen *et al.*, 1989; Voss *et al.*, 1992) or after transfection of eukaryotic expression vectors (Mergener *et al.*, 1992; Smith *et al.*, 1990).

The products of the *gag* and *pol* genes are translated as two polyproteins ($pr55^{gag}$ and $pr160^{gag\text{-}pol}$ in the case of HIV-1) whereby synthesis of the *pol*-derived proteins is achieved by translational frameshifting in the 3′ terminal part of the *gag* region (Jacks *et al.*, 1988). The $pr55^{gag}$ precursor can be divided into the four separate functional domains matrix (MA), capsid (CA), nucleocapsid (NC), and p6, which need to become proteolytically separated before the maturation of the virion. Cotranslational myristoylation of the N-terminal MA domain which is closely apposed to the inner surface of the lipid envelope of the virus (Gelderblom *et al.*, 1987) has been shown to be essential for the produc-

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tion of extracellular particles (Bryant and Ratner, 1990; Göttlinger *et al.*, 1989). It is likely, however, that besides N-terminal acylation, additional signals governing plasma membrane targeting reside on MA. This conclusion is supported by the observation that in type D retroviruses MA mutations profoundly affect the intracellular targeting of structural polyproteins (Rhee and Hunter, 1990, 1991). The HIV *env* gene also is expressed as a precursor protein, gp160, which undergoes proteolytic cleavage to yield a membrane-fusion-competent glycoprotein complex consisting of gp120 and gp41. Proteolytic cleavage is carried out by a cellular proteinase and requires the presence of a specific sequence of basic amino acids at the cleavage site. Cleavage is essential for the production of infectious HIV-1 (McCune *et al.*, 1988; Bosch and Pawlita, 1990). The requirements for specific incorporation of viral glycoproteins into virion particles are not understood in any viral system but it is likely that an interaction between the MA and TM proteins plays a role in this process. In the case of Rous sarcoma virus, a close proximity of TM and MA has been demonstrated by chemical cross-linking (Gebhardt *et al.*, 1984) and in frame deletions within the MA domain of HIV-1 have been shown to affect the incorporation of viral glycoproteins (Yu *et al.*, 1992). The CA protein (p24) forms the core shell of the virus particle which encases a ribonucleoprotein complex consisting of the dimeric viral RNA genome associated with the NC protein. The localization and role in assembly of the C-terminal p6 domain of the Gag polyprotein is not clear. It does not appear to be required for viral assembly but presumably plays an essential role in late stages of the budding process allowing for the release of viral particles (Göttlinger *et al.*, 1991). In mature HIV particles, p6 has been proposed to form a specific core–envelope link at the narrow end of the core (Höglund *et al.*, 1992).

Considerable progress has been made in recent years in identifying the location of individual proteins within the retroviral particle (Gelderblom, 1991). Nevertheless, the molecular processes involved in intracellular transport and accumulation of polyproteins, and the molecular interactions driving assembly, bud formation, and maturation remain poorly understood. Most of the studies on assembly of wild type and mutant HIV proteins have relied on transient expression systems. While such experiments are convenient for studying retrovirus morphogenesis and the contributions of specific domains on particle formation and release, they suffer from considerable variability between individual experiments and cannot easily be scaled up. A detailed biochemical analysis of intermolecular interactions in the viral particle and of interactions between viral and cellular proteins in the process of assembly would be greatly aided by the availability of cell lines stably producing virus-like particles. Moreover, such cell lines could also serve as good candidates for the production of recombinant HIV immunogens and as vehicles for the targeting of foreign genes into specific cells. In this study, we have analyzed protein expression and virus-like particle formation following coexpression of HIV-1 *gag* and *env* gene products from heterologous promoters, and have shown that stable production of significant amounts of particles depends on the absence of enzymatically active HIV PR.

MATERIALS AND METHODS

Cells and transfections

COS 7 cells and CV-1 cells were maintained in DMEM supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 m*M* glutamine. Transfections were performed as described previously (Bosch and Pawlita, 1990; Mergener *et al.*, 1992). Briefly, approximately 5 $\times$ 10^6 cells were suspended in 0.1 ml PBS and electrotransfected with 10–20 μg DNA using a Bio-Rad gene pulser. The electrical parameters were 150 V, 960 μF, 100 Ohm resistance for transfection of COS cells and 120 V, 960 μF without the use of the pulse controller for transfection of CV-1 cells.

Expression plasmids

Plasmids for the expression of HIV-1 *gag* and *pol* gene products have been described previously (Mergener *et al.*, 1992). Briefly, pK-R-gpII and pK-R-gpV contain a short stretch of 5′ untranslated sequence followed by the entire *gag* and PR (gpII; Kräusslich *et al.*, 1988) or *gag* and *pol* (gpV) regions and the *rev* responsive element (RRE) of HIV-1 (strain BH10; Ratner *et al.*, 1985) under control of the human cytomegalovirus immediate–early promoter/enhancer element. pK-R-gpIIa is a derivative of pK-R-gpII containing a mutation of the Asp25 codon of the PR active site to Ala25 (Mergener *et al.*, 1992). pK-R-gpIIΔFS was derived from pK-R-gpII by site-directed mutagenesis using the polymerase chain method and its construction will be described in detail elsewhere. Briefly, the HIV-1 frameshift signal sequence AATTTTTTAGGG (nt 1627–1635), where translational frameshifting occurs at the run of six U residues on the viral RNA (Jacks *et al.*, 1988), was changed to AAcTTccTgGGG (altered nucleotides in lower case) and the resulting plasmids were shown not to direct any synthesis of *pol* gene products after transfection of mammalian cells (M.F. and H.-G.K., unpublished observations). All *gag* expression vectors contain, in addition, the gene conferring resistance to hygromycin B driven by a herpes simplex thymidine kinase promoter. For expression of the HIV-1 Rev protein, plasmid pMTcrev (Hadzopoulou-Cladaras *et al.*, 1989) which contains the *rev* cDNA under control of

the metallothionin promoter as well as the neomycin resistance gene was used.

A SV40 late replacement vector, pLExHIVenv, for transient expression of HIV-1 *env*, has been described previously (Bosch and Pawlita, 1990). The inserted HIV-1 fragment (nucleotide positions 5818–8926 (strain BH10) contains, in addition to *env*, the genes for *tat*, *rev*, and *vpu*, all of which are expressed (Rekosh *et al.*, 1988; V.B. unpublished observation). To generate the plasmid pLβAcenv, the SV40 sequences, consisting of the T-antigen gene and the SV40 origin of replication in pLExHIVenv, were replaced by the 4.3-kb *Eco*RI to *Alu*I fragment from the human β-actin gene isolate p14T-β-17 (Leavitt *et al.*, 1984). The derivative pLβAcenv/neo, contains, in addition, the neomycin resistance gene under control of the herpes simplex thymidine kinase gene promoter and polyadenylation signals (Fig. 1).

Generation of stable HIV-1 protein-expressing cell lines

Stable cell lines were generated by cotransfection of linearized plasmids and subsequent selection with either 300–400 μg/ml neomycin, 50–200 μg/ml hygromycin, or both depending on the resistance marker used. Cell clones were expanded and analyzed for HIV-1 Gag protein expression using indirect immunofluorescence of cells and ELISA of culture media and for expression of HIV-1 glycoproteins by indirect immunofluorescence. Expression of the Rev protein was not analyzed directly but was deduced from the fact that structural protein production in the case of HIV is completely dependent on concomitant Rev protein expression. Positive cell clones were subcloned and were analyzed for homogeneity in indirect immunofluorescence and for stability of protein expression.

The cell line, Cl-4, was generated by cotransfection of pLβAcenv, linearized with *Eco*RI immediately 5′ to the β-actin promoter/enhancer sequence, together with pSV2-neo (Southern and Berg, 1982), in which the neomycin resistance gene is under the control of the SV40 early promoter and SV40 polyadenylation signals, linearized with *Eco*RI. Further wild-type HIV-1 glycoprotein-expressing cell lines (not shown), as well as a cell line coexpressing *env* and *gag* gene products (33/4; see below), were generated by transfection of pLβAcenv/neo, in which the neomycin resistance gene was present on the *env*-expressing plasmid. In these cases, pLβAcenv/neo was linearized with *Cla*I, which cleaves 5′ to the neomycin resistance cassette. All *gag* expression vectors (pK-R-gpII and derivatives) were linearized with *Nar*I which cleaves in the vector sequence 5′ to the HCMV-IE promoter. pMTcrev was linearized with *Eco*RI 5′ to the metallothionin promoter.

Analysis of expression products

For indirect immunofluorescence, cells were grown on sterile glass cover slips, washed in PBS, and fixed in methanol for 5 min at −20° followed by acetone for 5 min at −20°. For the detection of *gag* gene products, fixed cells were incubated with a polyclonal rabbit antiserum against HIV-1 CA (dilution 1:200; Mergener *et al.*, 1992); products of the *env* gene were reacted with a polyclonal rabbit antiserum against HIV-1 gp160 (dilution 1:100; Bosch and Pawlita, 1990). Immune complexes were detected with fluorescein isothiocyanate-labeled anti-rabbit IgG (1:100; Dianova, Hamburg).

Analysis of membrane fusion capacity of HIV glycoproteins was performed as previously described (Wilk *et al.*, 1992a,b; Bosch and Pfeiffer, 1992). Stable glycoprotein-expressing cells were plated onto cover slips and were overlaid with an approximately fivefold excess of $CD4^+$ HeLa cells. Syncytia formation was analyzed by indirect immunofluorescence using anti-gp160 serum after 24 hr cocultivation.

For detection of HIV antigens, media were cleared and appropriate dilutions were analyzed using a commercial ELISA kit (Organon Teknika, Eppelheim, Germany). For quantitative detection of processed CA antigen, virus-like particles were sedimented through a sucrose cushion, stripped of their lipid envelope with 0.2% Triton X-100 and incubated with purified HIV-1 PR. Appropriate dilutions were analyzed using a commercial ELISA kit (Cellular Products, Inc., Buffalo, NY) and were plotted against a CA standard curve.

For Western blot analysis, cell or particle extracts were separated on SDS–polyacrylamide gels containing 17.5% polyacrylamide (200:1 ratio acrylamide:*N,N*-methylenebisacrylamide) for detection of *gag* gene products and 8% polyacrylamide (40:1 ratio) for detection of *env* gene products. After transfer to nitrocellulose membranes (Schleicher & Schuell) by electroblotting, blots were reacted with polyclonal antisera against HIV proteins as indicated above and with alkaline phosphatase-conjugated second antiserum (Jackson Immunochemicals, Inc.).

Purification of extracellular particles

Media from confluent monolayers of stable cell lines were cleared by centrifugation at 200 *g* for 10 min and virus-like particles were either precipitated with polyethylene glycol 6000 (PEG) or centrifuged through a sucrose cushion as described (Mergener *et al.*, 1992). For PEG precipitation, supernatants were made 1 *M* NaCl and 7.5% PEG 6000 and incubated for 60 min at 0°. Precipitates were collected by centrifugation at 12,000 *g* for 15 min and lysed in gel loading buffer. Alternatively, supernatants were centrifuged through a cushion of 20% (w/v) sucrose at 120,000 *g* for 2 hr at 4°.

Southern blot analysis

The extraction of total cellular DNA, digestion with appropriate restriction enzymes, agarose gel electrophoresis, transfer to nylon membranes, and detection of vector fragments by hybridization to specific probes was carried out using standard protocols (Sambrook *et al.*, 1989). Briefly, confluent cultures of the established cell lines (approx. 5×10^6 cells) were washed once with PBS and scraped into 1 ml lysis buffer (3% sarcosyl, 0.07 *M* Tris/HCl, pH 8.0, 0.025 *M* EDTA) diluted 1:3 in TE. After digestion with 0.1 mg/ml Proteinase K for 1 hr at 37°, the cell lysates were extracted with phenol, phenol:chloroform:isoamylalcohol (25:24:1), and chloroform:isoamylalcohol (24:1). Nucleic acid was precipitated with isopropanol, washed with 70% ethanol, dried, and redissolved in 100–200 μl TE at 4° overnight. Ten micrograms of genomic DNA from each cell line were digested with *Bam*HI overnight in a buffer containing RNase A. The digested DNA samples were electrophoresed in a 1% agarose gel and transferred to Gene Screen Plus membranes using standard procedures. The fragment used to detect integrated HIV-1 *gag* sequences was prepared from *Eco*RI-digested pK-R-gpII and radioactively labeled with [α^{32}P]CTP by random priming. The position of this fragment, and the sizes and positions of the integrated sequences to which it can hybridize, are indicated in Fig. 7B. After exposure to film, the radioactive *gag* probe was washed off the filter with 0.4 *M* NaOH for 30 min at 42° and the filter rehybridized to a radioactive *env* probe prepared from *Sal*I plus *Xho*I-digested pLβAcenv plasmid. The position of the probe on the plasmid and the sizes and positions of the integrated fragments to which it can hybridize are indicated in Fig. 7B.

Electron microscopy

Subconfluent monolayers of stable cell lines were fixed with 2.5% glutaraldehyde in PBS for 30 min, carefully scraped off the plate, and centrifuged at 200 *g* for 5 min at 20°. Cell sediments were postfixed in 1% OsO_4, embedded in agar, treated with 1% tannic acid, and processed and embedded in Epon as described elsewhere (Gelderblom *et al.*, 1987). Sections of 40 to 60 nm in thickness were prepared from at least two replicate blocks from each specimen, poststained with lead citrate, and evaluated using a Zeiss electron microscope 10 A at 60 kV.

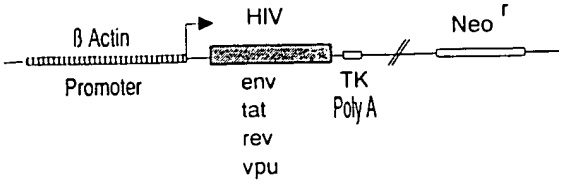
RESULTS

Transient coexpression of *gag* and *env* genes

Previously, we had shown that transient coexpression of HIV-1 *gag* and *rev* genes in mammalian cells leads to the synthesis of correctly processed viral structural polyproteins and efficient secretion of virus-like particles that were indistinguishable from immature virions except for the lack of glycoprotein spikes (Mergener *et al.*, 1992). To achieve the production of particles incorporating the viral glycoproteins, we performed cotransfection of COS 7 cells with the *gag* expression vector pK-R-gpII (Mergener *et al.*, 1992) which contains the HIV-1 *gag* and PR regions and the RRE under control of the HCMV-IE promoter/enhancer element, together with the *env* expression vector pLExHIVenv (Bosch and Pawlita, 1990) which also codes for the *tat, rev,* and *vpu* genes. Two days after transfection, cell-associated HIV CA protein and glycoproteins could be readily detected by indirect immunofluorescence or by immunoblot analysis of cell lysates (data not shown). Analysis of particulate fractions from culture media, obtained either by PEG precipitation or by sedimentation through a sucrose cushion, yielded large amounts of particle-associated CA protein, similar to what had been described previously (Mergener *et al.*, 1992). However, analysis of particulate fractions using antisera directed against viral glycoproteins showed only low amounts of envelope proteins gp120 and gp160 (data not shown). Since infectious HIV-1 contains only the surface glycoprotein gp120 and not gp160, we hypothesize that the low levels of Env proteins detected after transient coexpression are due, at least in part, to membranous fragments released from dead cells.

Generation of stable wild type HIV-1 glycoprotein-expressing cell line and analysis of subviral particle formation

The inefficient incorporation of glycoproteins into virus-like particles after transient transfection may be due to insufficient amounts of glycoprotein available for incorporation. To test this idea, we established the cell line CI-4 by transfection of an *env* expression vector into CV-1 cells followed by selection and subcloning as described under Materials and Methods. Indirect immunofluorescence with anti-gp160 serum revealed that 100% of the cells were fluorescence-positive (Fig. 1A), albeit at different relative intensities, whereas the parent CV-1 cells were negative (Fig. 1B). When CI-4 cells were cocultivated with $CD4^+$ HeLa cells as described previously (Wilk *et al.*, 1992a,b) and subsequently subjected to indirect immunofluorescence, syncytia were observed (Fig. 1C), demonstrating that the HIV-1 glycoproteins are transported to the cell surface and are capable of inducing membrane fusion. Moreover, the HIV Env glycoproteins, expressed in the CI-4 cells, are able to complement an *env*-defective provirus. To demonstrate complementation, the HIV-1 proviral construct pNL4-3Δenv, with a deletion in the *env* gene, was transfected into CI-4 cells and the resulting released virions were analyzed for their infectivity in permissive T lymphocytes (Wilk *et al.*, 1992a). In contrast to expression in CV-1 cells, from



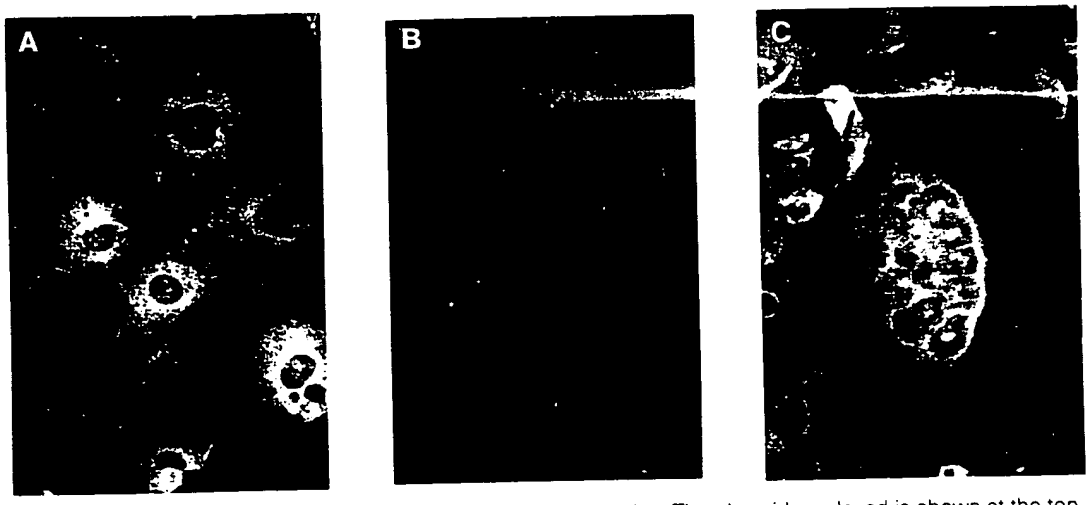


FIG. 1. Indirect immunofluorescence of CI-4 cells expressing HIV-1 glycoproteins. The plasmid employed is shown at the top. Expression of the HIV-1 genes is controlled by the human β-actin promoter/enhancer and the herpes simplex thymidine kinase gene polyadenylation signals (TK poly A). The plasmid is available with (pLβAcenv/neo) and without (pLβAcenv) a neomycin resistance gene expression cassette. (Bottom) Indirect immunofluorescence with a rabbit antiserum against gp160. (A) CI-4 cells, (B) CV-1 cells, and (C) coculture of CI-4 cells and CD4+ HeLa cells.

which noninfectious virus is released, transfection of pNL4-3Δenv into CI-4 cells resulted in the production of virus capable of a single round of infection in permissive MT-4 cells, as detected by indirect immunofluorescence and ELISA (data not shown). These results demonstrate that the HIV-1 glycoprotein in CI-4 is biologically fully active.

To demonstrate biochemically that CI-4 cells can produce virus-like particles that specifically incorporate HIV glycoproteins, we transfected the cells with plasmid pK-R-gpV which contains the complete HIV-1 *gag* and *pol* genes and the RRE (Mergener *et al.*, 1992). Cell lysates and PEG-precipitated particles were analyzed by immunoblotting 48 hr after transfection. Synthesis of the $pr55^{gag}$ polyprotein and its known cleavage products was observed in transfected cells but not in mock-transfected cells (Fig. 2a) and the transfected cells released virus-like particles containing the cleaved CA protein (Fig. 2a). The particle fraction also contained the viral glycoproteins gp120 and gp41, but not their cell-associated precursor gp160 (Fig. 2b). As expected, gp160, gp120, and gp41 were detected in lysates of mock-transfected and pK-R-gpV transfected CI-4 cells.

Establishment of stable cell lines expressing HIV Gag

To establish cell lines stably expressing the HIV-1 *gag* gene products and constitutively releasing mature virus-like particles, we performed cotransfection of COS 7 and CV-1 cells with plasmids pK-R-gpII or pK-R-gpV, together with the *rev* expression vector pMTcrev. Stable lines were selected as described under Materials and Methods and positive cell clones were initially detected by indirect immunofluorescence and ELISA and subsequently expanded. Gag protein expression was monitored by immunofluorescence and by immunoblot analysis of cell lysates and particulate fractions from culture media. All cell lines obtained after four independent transfections were highly unstable and became immunofluorescence-negative within several weeks to months after their initial establishment, even in the continuous presence of selective antibiotics (data not shown). The same result was also observed after repeated (at least three times) recloning of fluorescence-positive cell clones. Western blot analysis of fluorescence-positive cell lines consistently revealed low level expression of *gag*-derived proteins, which were mostly uncleaved (Fig. 3a). Very low levels of particles were detected and these particles consisted almost exclusively of uncleaved $pr55^{gag}$ (Fig. 3a). These results suggested that during outgrowth of the cell lines there was a selective pressure for loss of expression and for loss of PR function. To test this idea directly, CI-4 cells were transfected with pK-R-gpII and cell lysates derived from parallel transfections were analyzed for CA protein expression 2 and 40 days after transfec-

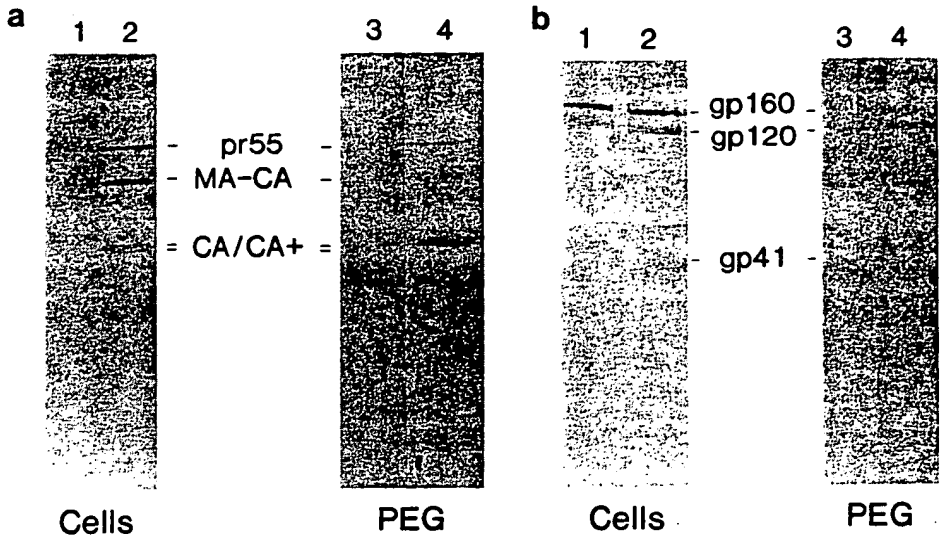


FIG. 2. Western blot analysis of *gag* and *env* gene products after transient transfection of CI-4 cells. Cells were either mock transfected (lanes 1 and 3) or transfected with plasmid pK-R-gpV (lanes 2 and 4). Cell lysates (Cells) and PEG precipitates of cleared media (PEG) harvested 48 hr after transfection were analyzed by immunoblotting. (a) Western blot was stained with antiserum against CA. (b) Western blot was stained with antiserum against gp160. HIV-specific precursor proteins and cleaved products are identified between the two panels. CA+ indicates heterogeneity in C-terminal processing, giving rise to several species of CA with slightly different electrophoretic mobilities.

tion (Fig. 3b). A dramatic difference was observed, with largely processed Gag proteins evident early after transfection and almost exclusively unprocessed $pr55^{gag}$ in the cell line CI-4:g/pIIef evident after 40 days. Lack of protein expression was not caused by the loss of HIV-derived integrated DNA since HIV *gag* gene-specific sequences could be readily detected as multiple copies by Southern blot analysis of fluorescence-negative and immunoblot-negative cells. In fact, the cell line CI-4:g/pIIef contains about 50 copies of the gag-specific sequence (Fig. 7; see below) but despite this, expression was very low.

Stable cell lines secreting immature Gag–Env particles

Since the selection of cell lines expressing wild type *gag* and *PR* genes yielded highly unstable lines producing predominantly uncleaved polyproteins, it appeared likely that there is strong selective pressure against the expression of active PR. We used two types of vectors to establish cell lines expressing wild type Gag polyproteins without proteolytically active PR. First, CI-4 cells were transfected with plasmid pK-R-gpIIa (Mergener *et al.*, 1992) containing a mutation of the active site *Asp*25 of PR to *Ala*25. Second, COS 7 cells were cotransfected with the *env* expression vector pLβAcenv/neo together with plasmid pK-R-gpIIΔFS, which is identical to pK-R-gpII except for a mutation in the sequence that mediates translational frameshifting from the *gag* to the *pol* open reading frame. This mutation, which does not change the amino acid sequence of the Gag polyprotein but destroys the run of six U-residues (on the transcribed RNA) known to be required for frameshifting (Jacks *et al.*, 1988), has been shown to completely abolish expression of the *pol* region (M.F. and H.-G.K., unpublished observation).

In both cases, positive cell clones could be readily detected by indirect immunofluorescence, and several cell lines stably expressing *gag* and *env* gene products were obtained after subcloning as described in Materials and Methods. Figure 4 shows immunofluorescence data for the two representative lines, 33/4 and CI-IX. The former was generated by coexpression of *gag* and *env* in COS cells (Figs. 4A and 4B), whereas the latter was established after transfection of CI-4 cells with pK-R-gpIIa (Fig. 4C and 4D). All cells show positive fluorescence with anti-gp160 serum (Figs. 4B and 4D) and with antiserum against CA (Figs. 4A and 4C) under conditions in which the parent CV-1 cells are negative (Figs. 4E and 1B). Both cell lines have been propagated for over 1 year and were shown to stably express both Gag and Env proteins, albeit with a slight reduction of expression levels after several months. Western blot analysis of fluorescence-positive cell lines revealed the expression of uncleaved $pr55^{gag}$ in 33/4 cells (Fig. 5a, lane 2), 32 cells (Fig. 5a, lane 1; another clone obtained after cotransfection of COS cells), and CI-IX cells (lane 4), but not in the parent COS 7 cells (lane 3). Moreover, we observed the release of virus-like particles in all cases (Fig. 5b) and the particles obtained consisted either of only uncleaved Gag polyproteins in the case of the ΔFS mutant (Fig. 5b, lanes 1 and 2) or of Gag and Gag–PR polyproteins in the case of inactive PR (Fig. 5b, lane 4). Particle release was analyzed quantitatively by ELISA of particulate frac-

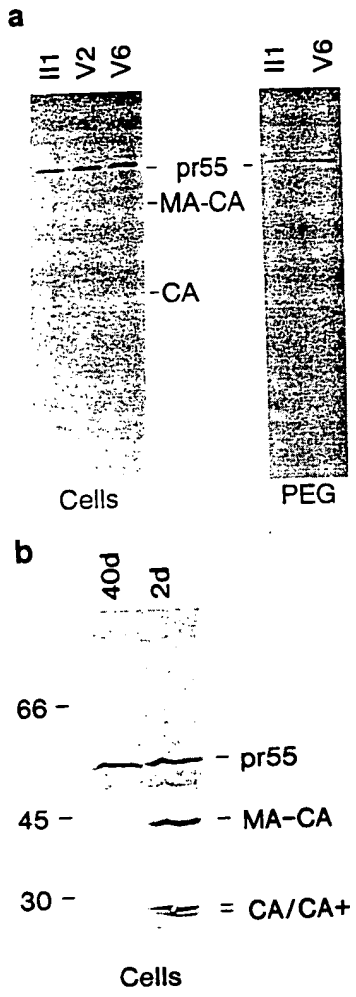


FIG. 3. Western blot analysis of *gag* gene products synthesized in stable cell lines and after transient transfection. Cell lysates (Cells) and PEG precipitates of cleared media (PEG) were resolved by SDS–PAGE and Western blots were stained with rabbit polyclonal serum against CA. (a) Immunoblot of cell lysates and particles derived from stable cell lines selected after cotransfection of COS 7 cells with plasmids pK-R-gpII (lane II1) or pK-R-gpV (lanes V2 and V6) together with pMTcrev as described under Materials and Methods. (b) Immunoblot of lysates of CI-4 cells 2 or 40 days after transfection with plasmid pK-R-gpII. HIV-specific precursor proteins and cleaved *gag* products are identified.

tions derived from culture media. The ELISA kit used detects primarily the processed CA antigen and we therefore incubated the sedimented and detergent-stripped particles with purified HIV-1 PR. Complete cleavage of the Gag polyprotein was confirmed by immunoblot analysis (data not shown) and relevant dilutions of the incubation mixture were analyzed using a quantitative ELISA. Analysis of particle fractions repeatedly yielded a CA antigen concentration of approximately 10–50 ng/ml of cell culture medium, corresponding to approximately 0.2 to 1 μg of Gag protein per 10^6 cells per day.

Immunoblot analysis of stable lines with anti-gp160 serum revealed the specific expression of the glycoprotein precursor gp160 and its cleavage products gp120 and gp41 in 33/4 cells (Fig. 5c) and in CI-4 cells (Fig. 2b). Analysis of virus-like particles, on the other hand, yielded only the particle-associated glycoproteins gp120 and gp41 (Fig. 5d, lanes 1 and 2), similar to the situation observed with wild type HIV-1. This observation is also supported by the results of thin section electron microscopy which showed typical lentiviral budding structures at the plasma membrane with envelope projections radiating from the surface of the bud (Fig. 6c). Released extracellular particles, on the other hand, did not exhibit the typical spherical structure of immature virions but showed an incomplete assembly of the ribonucleoprotein shell (Figs. 6a, 6b), similar to that observed previously with a PR-minus mutant (Mergener *et al.*, 1992). Particles were mostly devoid of envelope projections, indicating that, although glycoproteins were detected in released particles by Western blot analysis, glycoprotein shedding, which has been observed with infectious HIV (Gelderblom *et al.*, 1985), also occurred in this system (Fig. 6).

Southern blot analysis

To more fully characterize the *gag*- and *env*-expressing cell lines, we performed semi-quantitative Southern blotting using *gag*- and *env*-specific probes. Since the plasmids were linearized prior to transfection, we expected the DNA in the cell lines to be integrated by recombination at or near their free ends. Figure 7B shows the linearized plasmids as they are expected to be integrated. The arrows indicate the cleavage sites for *Bam*HI, which was used for digestion of the plasmid and cellular DNAs. Also shown are the positions and sizes of the fragments to which the *gag* probe and the *env* probe can hybridize. Digestion of pK-R-gpII with *Bam*HI generates a fragment of 2.8 kb, consisting of the *gag* and RRE sequences. Analysis of the cell lines transfected with derivatives of pK-R-gpII (designated gpII* in Fig. 7B) demonstrated the presence of specific fragments of this size (Fig. 7A, left). From comparison with the 1 copy and 10 copies standards, it can be seen that the integrated sequences are present in more than 1 copy per genome. The cell lines 33/4, CI-IX, and CI-4:g/pIIef contain about 2, 5, and 50 copies of integrated gpII* DNA, respectively (Fig. 7A, left).

The results of hybridization with the *env* probe are shown in Fig. 7A (right). Whereas the HIV *env*-specific insert was identical in all plasmids, the cell lines CI-4 and CI-IX were generated from plasmid pLβAcenv, and the line 33/4 was generated from pLβAcenv/neo containing different flanking plasmid sequences (Fig. 7B). In the cases of CI-4 and CI-IX cells, digestion with *Bam*HI should generate two fragments detectable with this probe (Fig. 7B). A weakly hybridizing fragment of 4.1 kb derived from the distal portion of *env* contains

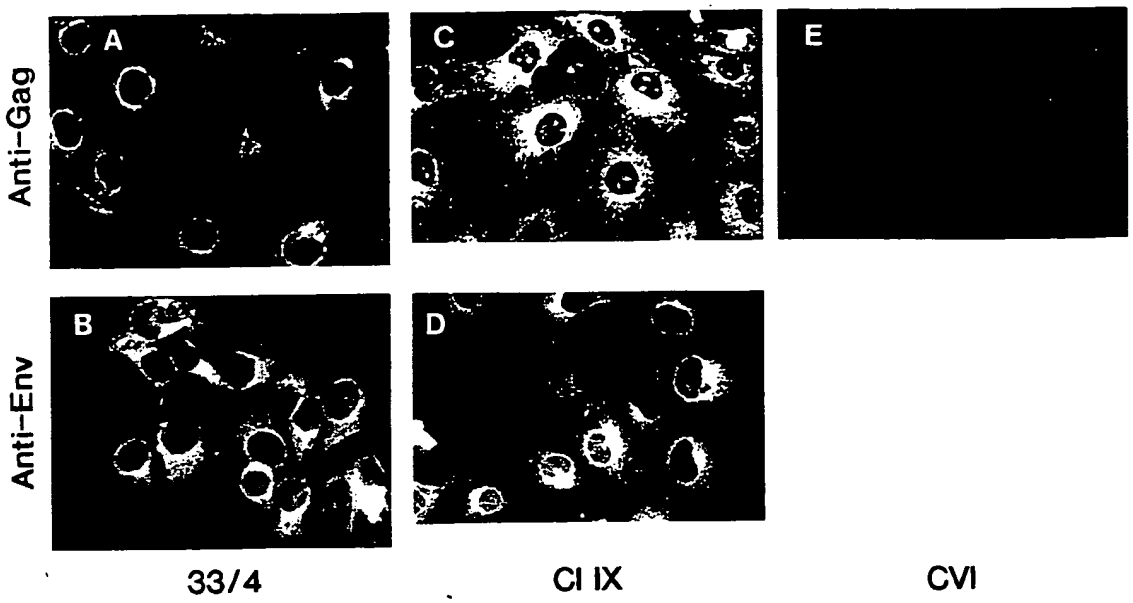


FIG. 4. Indirect immunofluorescence of stable cell lines 33/4 (A and B), CI-IX (C and D), and CV-1 cells (E). Fixed cells were probed with a polyclonal antiserum against CA (A, C, E) or against gp160 (B, D) followed by fluorescein isothiocyanate-labeled second antiserum.

only about 400 nt of *env*-specific sequence plus flanking plasmid sequences. A strongly hybridizing fragment of at least 7 kb contains most of the *env* gene, flanking plasmid sequences, plus flanking cellular sequences. The number of bands larger than 7 kb gives a lower estimate of the number of integration sites. In fact, three to four such fragments were observed (Fig. 7A). The *env* probe also detects the 2.8-kb *gag* fragment, since the RRE element cloned downstream of *gag* is derived from the middle of the *env* gene. A similar analysis of the cell line 33/4 yielded a strongly hybridizing band at about 9 kb representing most of the HIV *env* gene plus 5′ flanking plasmid sequences and three additional fragments larger than 5 kb which contain cellular flanking sequences. These fragments presumably represent three integration sites (Fig. 7A).

DISCUSSION

In the last few years, several reports have demonstrated that transient expression of lentiviral *gag* gene products from recombinant vectors, together with or without additional segments of the viral genome, can lead to the release of virus-like particles with the morphology of the immature or mature virion. Most of these studies were performed using virus-based vector systems, including vaccinia virus (Haffar *et al.*, 1990; Hu *et al.*, 1990; Karacostas *et al.*, 1989; Shioda and Shibuta, 1990; Voss *et al.*, 1992) or other poxvirus (Jenkins *et al.*, 1991) as well as baculovirus vectors (Gheysen *et al.*, 1989; Royer *et al.*, 1992). In a previous study (Mergener *et al.*, 1992), we examined virus-like particle formation following transient expression in COS cells of mammalian expression vectors encoding different wild type and mutated regions of the HIV-1 genome under control of a heterologous promoter.

The aim of the present study was to establish cell lines stably expressing wild type and mutated HIV-1 structural proteins, alone or in combination, and releasing virus-like particles into the culture medium. Such cell lines should be useful for structural and biochemical analyses of interactions important during viral assembly. Moreover, noninfectious virus-like particles have been proposed as immunogens for vaccination (Haffar *et al.*, 1991; Haynes *et al.*, 1991; Vzorov *et al.*, 1991; Rovinski *et al.*, 1992) and recombinant HIV-like particles have been shown to inhibit virus production from peripheral blood cells of seropositive donors (Haffar *et al.*, 1992). Cell lines constitutively expressing HIV structural proteins may also be of utility as packaging lines for the targeting of heterologous RNAs into $CD4^+$ T-cells. While the expression of *gag*-derived proteins alone, in the absence of other viral gene products, is sufficient for particle formation, PR-mediated polyprotein cleavage is required for morphological conversion of an immature virion to a mature virion which is likely to be important for subsequent uncoating of the virion in the target cell.

Our repeated efforts to isolate cell lines stably producing virus-like particles following transfection with expression vectors encoding wild type *gag* and *PR* sequences resulted in numerous antibiotic-resistant cell clones. However, these clones produced only low amounts of viral protein. Western blot analysis of cells and released particles revealed that not only was the expression of structural proteins low, but the proteins produced were almost completely uncleaved, indicat-

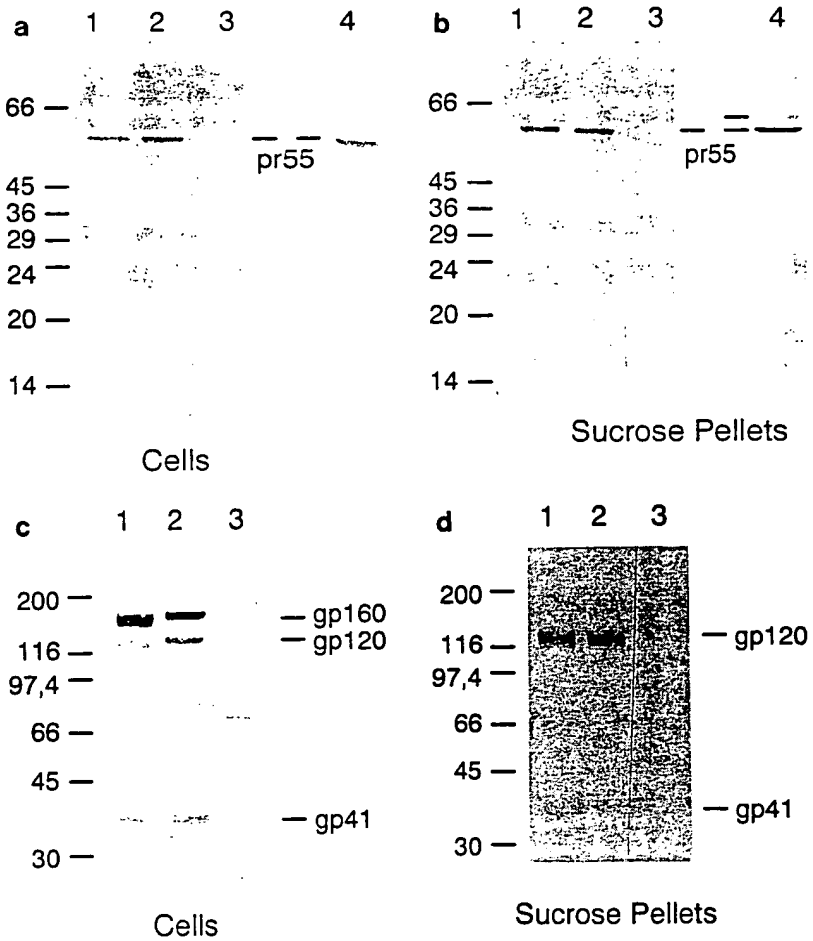


FIG. 5. Western blot analysis of *gag* and *env* gene products synthesized in and released from stably expressing cell lines. Cell lysates (a and c; Cells) or virus-like particles obtained by sedimentation through a sucrose cushion (b and d; Sucrose Pellets) were resolved by SDS-PAGE and Western blots were stained with polyclonal antisera against CA (a and b) or against gp160 (c and d). Lanes (1) 32 cells, (2) 33/4 cells, (3) COS 7 cells, (4) CI-IX cells. Molecular mass standards (in kDa) are indicated on the left; HIV-specific precursor proteins and cleavage products are identified on the right.

ing that there may be a selective disadvantage for those cells that overexpress active PR. Lack of intracellular processing may be due, at least in part, to the selection for low expression lines and consequent lack of intracellular dimerization of Gag-Pol polyproteins, which is required for PR activation. However, since the concentration of Gag and Gag-Pol proteins in particles should be independent of the intracellular protein concentration, this hypothesis cannot explain the virtual absence of proteolytic processing in particles. Possible explanations for this phenotype include selection of cells that downregulate PR activity or that express reduced levels of Gag-Pol due to an altered frameshifting efficiency, and thus yield lower amounts of active PR. Our results agree with a previous study (Haynes *et al.*, 1991) showing that constitutive expression of wild type HIV proteins in COS cells leads to release of low amounts of morphologically immature particles, whereas stable expression under control of an inducible promoter yields efficient production of mature particles. Unfortunately, in this study the relative amounts of processed versus unprocessed antigen were not analyzed in the different cell lines generated.

It is likely that the observed selection against stable expression of enzymatically active PR is due to the cleavage of cellular proteins by HIV-1 PR. Previously, it has been shown that overexpression of PR, either by mutation of the frameshift signal (Mergener *et al.*, 1992) or by expression of a genetically linked dimer of HIV-1 PR as component of the viral polyprotein (Kräusslich, 1991), leads to loss of particle formation and rapid cell killing. Experiments with specific PR inhibitors have demonstrated that this phenotype is caused by PR activity directly (Kräusslich, 1992), presumably because of PR-mediated proteolytic degradation of cellular proteins (e.g., vimentin; Shoeman *et al.*, 1990). Moreover, analysis of acutely and permanently HIV-1-infected T-cells revealed high levels of PR activity in the acutely infected cells only and accumulation of almost completely uncleaved polyproteins in a persistently infected cell line (Kaplan and Swanstrom, 1991). Consistent with these findings, we could easily

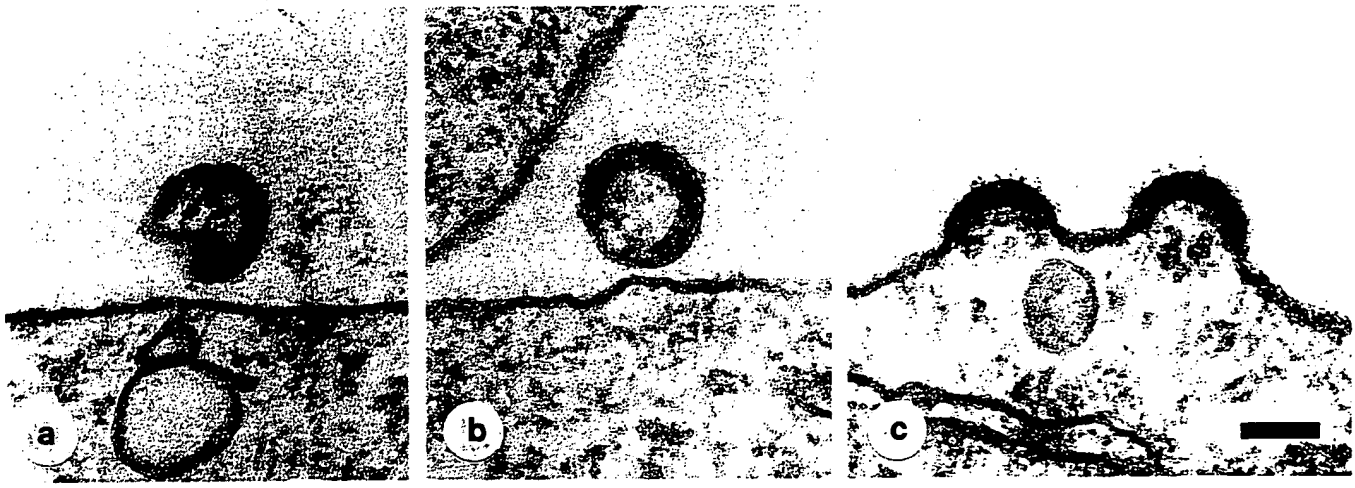


FIG. 6. Thin section electron microscopy of HIV-like particles produced from cell line 33/4 showing cell-released (a, b) and budding structures (c). Both extracellular (a, b) particles reveal unfinished core–shell formation and occasional surface knobs on the lipid bilayer. (b) Two incomplete shells adjacent within a single particle. (c) The two crescents depicted show all morphological details typical of early lentivirus assembly: the 20-nm thick ribonucleoprotein shell is closely attached to the lipid bilayer with the viral RNA as a more electron dense layer at its inner leaflet. Viral glycoprotein knobs are sparsely distributed on the protruding particles. Magnification × 100,000; Bar = 100 nm.

establish stable cell lines producing uncleaved HIV-1 Gag proteins with or without additional segments of the HIV-1 genome, but not expressing enzymatically active PR. These cells constitutively release virus-like particles containing the $pr55^{gag}$ precursor protein and wild type HIV-1 envelope glycoproteins.

Electron micrographs of cells stably expressing PR-defective particles revealed regular lentiviral budding structures at the plasma membrane but released particles did not exhibit the typical spherical core of immature retroviruses. Instead, in thin sections they displayed incompletely closed ring structures or even multiple incompletely assembled core shells in a single particle. These results are in agreement with previous observations regarding particle morphology after expression of a PR-minus provirus (Peng *et al.*, 1989; H.-G.K., unpublished observations) or of subviral constructs expressing functionally inactive PR (Mergener *et al.*, 1992), as well as with electron microscopic analysis of immature HIV-1 particles released from infected cells treated with specific inhibitors of PR (Schätzl *et al.*, 1991). While some immature particles with a small gap in the ring structure have also been observed on electron micrographs of a PR-defective avian retrovirus (Stewart *et al.*, 1990), PR-defective HIV particles display a much more pronounced morphological alteration of the immature core shell and this alteration is found in all particles but not in wild type controls which were analyzed in parallel. We interpret these results as evidence for a requirement of HIV PR activity prior to formation of the immature spherical core. This activity may be directed against HIV polyproteins or against cellular proteins at the assembly site. This hypothesis may also explain the relative leakiness of intracellular HIV PR activation (Kaplan and Swanstrom, 1991) when compared with, e.g., avian retroviruses (Eisenman *et al.*, 1975; Stewart *et al.*, 1990).

Specific incorporation of viral glycoprotein is an important and poorly understood step in the production of infectious virus. We analyzed this process in particles released after transient and stable coexpression of Gag and Env proteins. Following transient transfection, low amounts of gp160 and gp120 were detected in the particulate fraction. We interpret the presence of the uncleaved gp160 as evidence for a contamination of the particles with membranous vesicles since infectious virions show only gp120. While some previous reports studying transient coexpression of *gag* and *env* gene products clearly showed the presence of only the processed glycoproteins in partially purified particles (Haffar *et al.*, 1991; Haynes *et al.*, 1991), others demonstrated both precursor and processed glycoproteins in particulate fractions after coexpression of SIV *gag* and *env* gene products (Jenkins *et al.*, 1991), again raising the question as to whether these glycoproteins are truly incorporated into virus-like particles. The reason for the failure in attaining glycoprotein incorporation in some studies in comparison to the other reports and in comparison to the situation in which *env* is stably expressed (Haynes *et al.*, 1991; this study) is unknown but may be explained by a requirement for a specific regulation of *gag* and *env* gene expression and transport which may not be accurately reproduced in some experimental systems. Specific incorporation of only the processed glycoproteins into virus-like particles was observed in cell lines stably coexpressing *gag* and *env* gene products or following transient expression of *gag* gene products in cell lines constitutively expressing wild type HIV-1 glycoproteins. The expression of functional glycoprotein in these cells was confirmed by

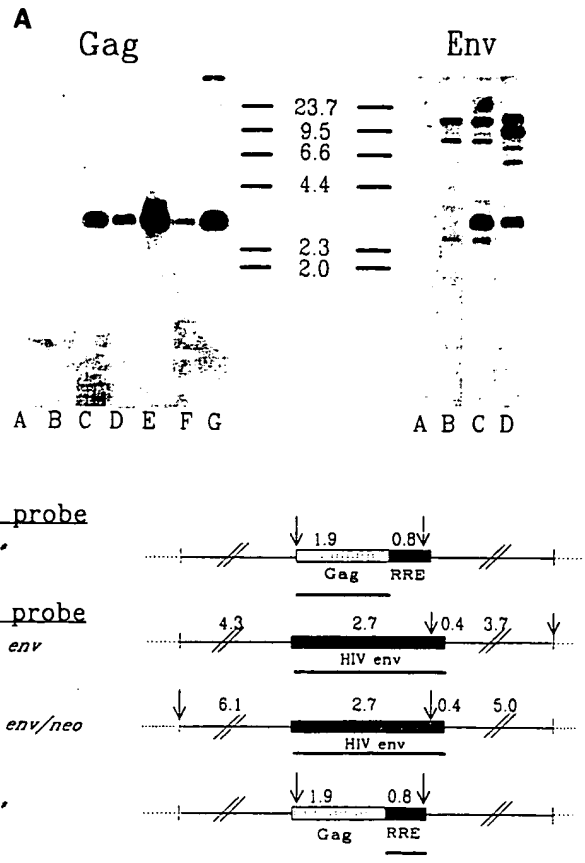


FIG. 7. Southern blot analysis of stable cell lines expressing HIV-1 *env* and *gag* gene products. (A) Gag, hybridization of *Bam*HI-digested cellular DNA with the *gag* probe. Lane A, CV-1 cells; B, CI-4 cells; C, CI-IX cells; D, 33/4 cells; E, CI-4:g/pIIef cells; F, 1 copy per cell plasmid pK-R-gpII mixed with CV-1 DNA; G, 10 copies per cell plasmid pK-R-gpII mixed with CV-1 DNA. Env, hybridization of *Bam*HI-digested cellular DNA with the *env* probe. Lane A, CV-1 cells; B, CI-4 cells; C, CI-IX cells; D, 33/4 cells. (B) Schematic picture of the relevant inserts from plasmids, pK-R-gpII and derivatives (designated g/pII* since the vectors used contain the same *gag*-specific insert with different point mutations), pLβAcenv and pLβAcenv/neo as they should be present in the integrated form. The vertical arrows denote the positions of the *Bam*HI sites. The stippled and solid boxes correspond to HIV-1 *gag* sequences and *env* sequences, respectively. Flanking solid lines designate plasmid sequences, whereas the dotted lines correspond to flanking cellular sequences. The numbers above each line give the sizes of the respective segments derived from the HIV-specific insert and flanking plasmid sequences and the bold horizontal lines below demarcate the sequences to which the respective HIV-specific probes can hybridize.

the formation of syncytia after cocultivation with CD4$^+$ HeLa cells and the ability to complement a proviral construct with defective *env* gene.

It is of interest to compare our newly generated cell lines with previously established mammalian cell lines stably expressing HIV-1 structural proteins. Constitutive production of HIV-1 glycoproteins has been reported for two CD4$^+$ T-lymphocyte cell lines (Kawamura *et al.*, 1989; Stevenson *et al.*, 1988) and for CHO and HeLa cells (Pitts *et al.*, 1991; Gama Sosa *et al.*, 1989). However, the T-cell lines may not be suitable for all applications because T-cells are poorly transfectable. Furthermore, the *env*-expressing cell lines described by Kawamura *et al.* (1989) do not produce authentically processed Env proteins. To date, there has been only a single report describing the stable coexpression of HIV *gag* and *env* gene products in mammalian cells (Haynes *et al.*, 1991). These authors used a construct containing almost the entire HIV-1 genome with a deletion of the LTRs under control of heterologous promoters. In this case, high-level expression of HIV structural proteins depended on inducible control of viral protein expression. The authors reported a very high yield of cell-released HIV-like particles (0.6 mg/ml p24 antigen) upon induction, which was constant for a period of 5 days. Given the observed toxicity of active PR, however, it appears likely that longer periods of induction would not be compatible with cell viability, whereas in the cells described in this report, constitutive secretion of virus-like particles can be observed over long periods of time. Moreover, release of immature particles containing only uncleaved pr55^{gag} also may be advantageous because of the enhanced stability of immature cores compared with that of mature cores (Stewart *et al.*, 1990). By contrast, generation of cell lines releasing mature Gag–Env particles will be required for the development of HIV-based retroviral vector systems specific for CD4$^+$ cells. Besides the use of inducible promoters, such cell lines may also be produced by use of PR mutants with reduced cytotoxic effects, selection of cell lines exhibiting reduced sensitivity to PR-mediated toxicity, or selection of cell lines in the presence of specific inhibitors of PR.

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Molecularly Cloned Simian Immunodeficiency Virus SIVagm3 Is Highly Divergent from Other SIVagm Isolates and Is Biologically Active In Vitro and In Vivo

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Simian immunodeficiency viruses have been isolated from African green monkeys originating from Ethiopia. A molecular clone, termed SIVagm3, was found to be highly divergent from SIVagmTYO-1 in terms of its restriction map and partial nucleotide sequence. A premature stop codon present in the transmembrane protein of SIVagm TYO-1 was absent in SIVagm3. SIVagm3 was biologically active in vitro and in vivo and displayed characteristics reminiscent of the wild-type virus. Biological activity was demonstrated by seroconversion of juvenile African green monkeys and *Macaca nemestrina* after inoculation. In contrast to antibody reactivity mainly directed against *env* proteins in naturally infected African green monkeys, African green monkeys and *M. nemestrina* infected with the cloned virus showed antibody reactivity directed against all major proteins as demonstrated by immunoblot analysis. The availability of a biologically fully competent molecular clone of SIVagm allows us now to address various pertinent questions in an animal model system which should help to understand features of human immunodeficiency virus infection in human beings.

Immunodeficiency viruses related to human immunodeficiency virus (HIV) have been isolated from several monkey species. Simian immunodeficiency virus (SIV) from mandrills (SIVmnd) has been isolated and characterized (20). This new member of the SIV-HIV group seems to be equally distinct from all immunodeficiency viruses isolated so far. Isolates from macaques (SIVmac) are now relatively well characterized and may be useful as animal models for HIV infection and disease induction (2, 15). SIVmac is much more closely related to HIV type 2 (HIV-2) than to HIV-1 (3, 7), and there is increasing evidence that some HIV-2 isolates are also infectious for macaques (6, 12). The origin of SIVmac is as yet unknown because of the remarkable similarity of independent isolates to each other and because of the apparent lack of anti-SIV antibodies in wild macaques (14). These data indicate a quite recent infection of captive macaque populations with a virus closely related to HIV-2. HIV-related viruses (SIVagm) from African green monkeys (AGMs) have been isolated by us (11) and others (5, 16). The complete nucleotide sequence of one isolate, termed SIVagmTYO-1, has been reported (8). SIVagm, like HIV and SIVmac, uses the CD4 molecule as a cellular receptor. SIVagm is cytopathic for permissive cells but replicates poorly, if at all, in many CD4-positive T-cell lines (11). Among AGMs in the wild, 30 to 40% are seropositive for SIVagm (9). Infected AGMs are apparently healthy, and it is unknown whether SIVagm is per se apathogenic or whether its apathogenicity is based on a special kind of evolved host-virus relationship. The possible pathogenicity of SIVagm in other monkey species is under investigation in our laboratory. Such studies may provide insight into the pathogenesis of HIVs and the evolution of lentiviruses. Therefore analysis of functional molecular SIVagm clones is essential for determining the variability of SIVagm and for exploring the molecular basis for the apathogenicity of these lentiviruses in their natural hosts.

We report here the characterization of a replication-competent molecular SIVagm clone, termed SIVagm3. SIVagm3 is infectious in vitro and elicits strong antibody response in vivo after infection of AGMs and pig-tailed macaques, from both of which it can be reisolated.

MATERIALS AND METHODS

Virus. Virus isolation from animals housed in our colony and subsequent growth in Molt-4 clone 8 cells (Molt-4/8 cells; kindly provided by M. Hayami, Kyoto) have been described previously (11).

Molecular cloning. Hirt supernatant DNA, prepared 48 h after infection of Molt-4/8 cells with SIVagm3 at high multiplicity, was digested with *Eco*RI and ligated into the *Eco*RI arms of lambda gtWES-lambda B. The resulting library was screened with the nick-translated HIV-1-derived *gag-pol* clone BH5 (19) under conditions of low stringency (25% formamide, 0.8 M NaCl, 37°C). The nylon filters (Hybond N; Amersham Corp.) were hybridized for 30 h, washed with 5× SSC (1× SSC is 0.15 M NaCl–0.015 M sodium citrate) at 50°C, and exposed to Kodak XAR films. Positive plaques were isolated and rescreened. The remaining positive plaques were analyzed by preparation of the phage DNA and subsequent Southern blot analysis after digestion with various enzymes. A 10-kilobase *Eco*RI fragment containing full-length viral DNA, permuted at a single *Eco*RI site in the *env* gene, was cloned into pUC18 to generate plasmid pSIVMB1. Restriction enzyme mapping was performed by hybridization of subgenomic HIV-1- and HIV-2-derived fragments to multiple digests of pSIVMB1 DNA.

Nucleotide sequence analysis. Appropriate restriction enzyme fragments were subcloned into the Bluescript vector (Stratagene). DNA sequences were determined from plasmid DNA minipreparations by the dideoxy-chain termina-

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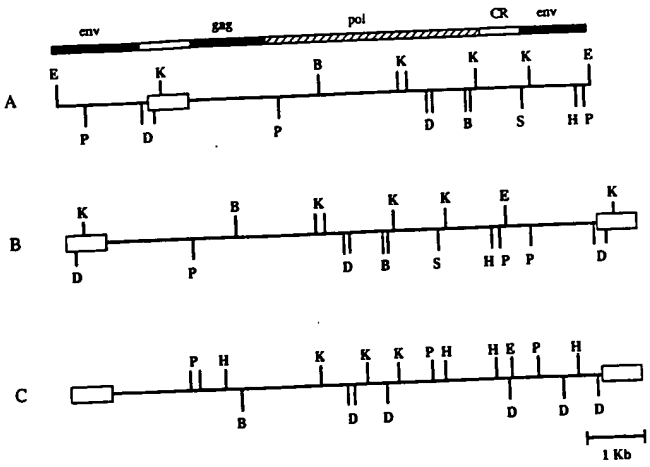


FIG. 1. Comparison of restriction maps of SIVagm3 and SIVagm TYO-1. (A) Restriction map of the permuted *Eco*RI fragment cloned from closed circular viral DNA present in Hirt supernatant of wild-type SIVagm3-infected cells. (B) Restriction map of a SIVagm3 provirus, deduced from the mapped *Eco*RI fragment. (C) Restriction map of SIVagmTYO-1. The map was generated from the published nucleotide sequence (5). Open boxes represent long terminal repeats. Abbreviations: B, *Bam*HI; D, *Dra*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sst*I; Kb, kilobases.

tion method (18) with Sequenase (U.S. Biochemical Corp.). Sequence data were analyzed by using the Microgenie program (Beckmann).

DNA transfection. A 10-μg sample of plasmid pSIVMB1 DNA was digested with *Eco*RI and subjected to agarose gel electrophoresis. Viral DNA was extracted from the gel and self-ligated to obtain concatemeric forms. The ligation mixture was extracted with chloroform, ethanol precipitated, dissolved in 10 μl of water, and transfected into Molt-4/8 cells in the presence of DEAE-dextran as previously described (15). Immunoblotting and reverse transcriptase (RT) assays were performed by published methods (11).

RESULTS

For a detailed analysis of a simian retrovirus isolate from an AGM in our colony (Paul-Ehrlich-Institut), Southern blot analysis of nonchromosomal DNA prepared from freshly infected Molt-4/8 cells was performed. Digestion with *Eco*RI resulted in a faint band of approximately 10 kilobases, presumably from single-cut circular DNA. Two predominant bands of smaller size from single-cut linear DNA were also observed (data not shown). *Eco*RI-digested Hirt DNA larger than 9 kilobases was pooled and ligated into the *Eco*RI arms of lambda gtWES-lambda B. The resulting library was screened by use of a HIV-1 derived *gag*-specific probe under low-stringency conditions. A full-length molecular clone termed SIVagm3 was isolated and subjected to further analysis after molecular cloning into pUC18.

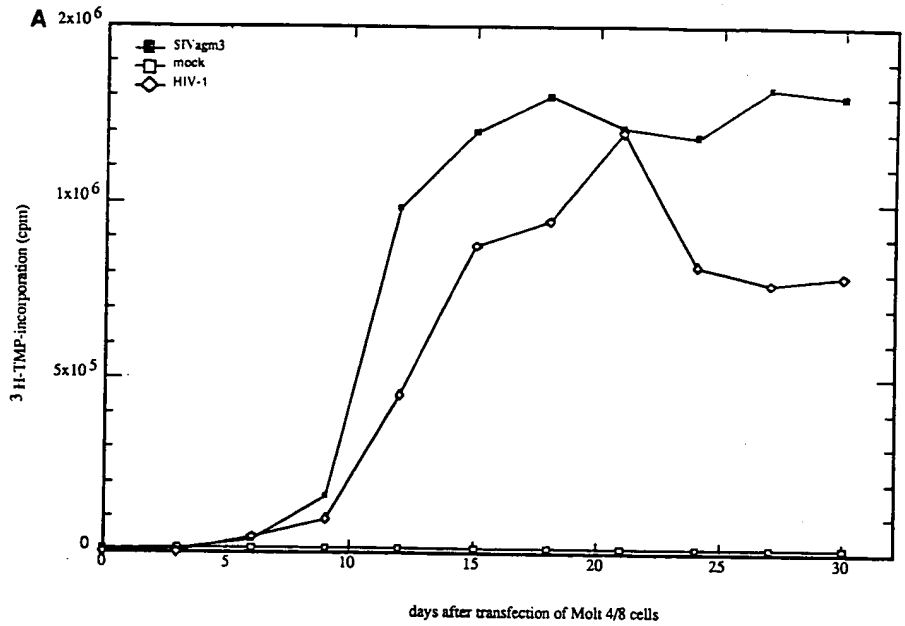
Restriction mapping and Southern blot analysis with *gag*-, *pol*-, *env*-, and long terminal repeat-specific probes derived from molecular clones of HIV-1 and HIV-2 revealed the overall organization of viral DNA from the cloned SIVagm3. The viral DNA was permuted at an internal *Eco*RI site in the *env* region (Fig. 1). A single long terminal repeat was located at the 5′ end. SIVamg3 thus represents a single-cut circular episome with one long terminal repeat of a simian lentivirus from AGMs.

In Fig. 1a, a detailed restriction map is shown compared with a map of SIVagmTYO-1 deduced from its published nucleotide sequence (8). Restriction enzyme site diversity was 65% between both AGM viruses, calculated as described by Saag et al. (17).

To substantiate the diversity between SIVagm3 and SIVagmTYO-1, we determined the nucleotide sequence of a part of the SIVagm3 *env* gene. A characteristic premature stop codon is present in the published nucleotide sequence of the SIVagmTYO-1 *env* gene (8). For nucleotide sequencing, it was therefore of special interest to select the part of the *env* gene corresponding to the coding region of the transmembrane protein (TMP). An alignment of the deduced amino acid sequences of SIVagm3 and SIVagm TYO-1 TMP

```
                1
SIVagm3     SerTrpPheAspPheSerLysTrpLeuAsnIleLeuLysIleGlyPheLeuAspValLeu
SIVagmTYO-1  *  *  *  *  *  *  *  *  *  *  *  * Met *  *  * ValIleVal

               21
SIVagm3     GlyIleIleGlyLeuArgLeuLeuTyrThrValTyrSerCysIleAlaArgValArgGln
SIVagmTYO-1  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  * Gly *  * Val *

               41
SIVagm3     GlyTyrSerProLeuSerProGlnIleHisIleHisProTrp - LysGlyGlnProAsp
SIVagmTYO-1  *  * Val *  *  *  *  *  *  *  *  * GlnValGly *  * Arg *  *

               60
SIVagm3     AsnAlaGluGlyProGlyGluGlyGlyAspLysArgLysAsnSerSerGluProTrpGln
SIVagmTYO-1  *  * AspGlu *  *  *  *  *  * AsnSerArgIleLysLeu * Ser * End

               80
SIVagm3     LysGluSerGlyThrAlaGluTrpLysSerAsnTrpCysLysArgLeuThrAsnTrpCys
SIVagmTYO-1  * Asp *  -  -  -  -  -  *  *  *  -  * MetGln *  * Ala * Leu

              100
SIVagm3     SerIleSerSerIleTrpLeuTyrAsnSerCysLeuThrLeuLeuValHisLeuArgSer
SIVagmTYO-1 ThrArgLeuAsnThr *  *  *  *  *  *  *  *  *  *  * IleGln *  * Lys

              120
SIVagm3     AlaPheGlnTyrIleGlnTyrGlyLeuGlyGluLeuLysAlaAlaAlaGlnGluAlaVal
SIVagmTYO-1  *  *  *  * Leu *  *  *  * Ala *  *  * ThrGly *  *  * IleLeu
```

FIG. 2. Predicted partial amino acid sequence of the TMP of SIVagm3 and alignment with the equivalent coding region of SIVagmTYO-1. The amino acids of SIVagmTYO-1 correspond to nucleotide 7840 through 8241 of the published nucleotide sequence. Asterisks mark similar amino acids; bars represent gaps introduced for optimized alignment.



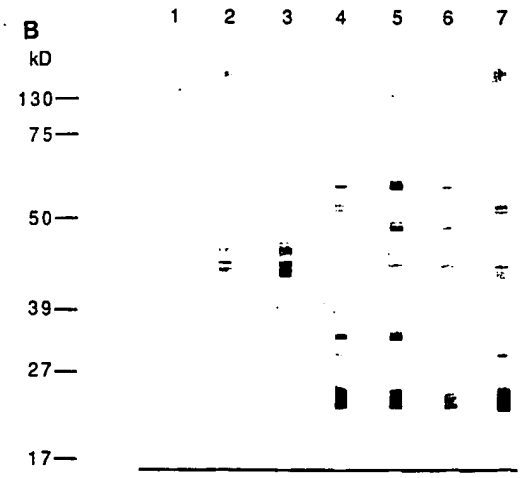


FIG. 3. (A) Time course of RT activity after transfection of molecularly cloned SIVagm3 DNA into Molt-4/8 cells. (B) Western blot analysis of viral proteins prepared from supernatant of transfected cells. Lanes: 1, AGM serum negative for SIVagm; 2 and 3, sera from AGMs positive for SIVagm; 4, human serum positive for HIV-1; 5 and 6, human sera positive for HIV-2; 7, *M. nemestrina* serum positive for SIVagm. kD, Kilodaltons.

regions is shown in Fig. 2. An amino acid identity of 67% was found. The premature stop codon previously found in the coding region of the TMP of SIVagmTYO-1 is not present in SIVagm3. We concluded that our isolate is an authentic but quite diverse member of the SIVagm group.

To test the biological activity of SIVagm3, we transfected the full-length *Eco*RI fragment of SIVagm3 after self-ligation into Molt-4/8 cells. RT activity was detectable in the cell supernatant within 8 days after transfection (Fig. 3A); although the initially extensive syncitium formation decreased, the level of RT activity was sustained. The supernatant of transfected cells was filtered and used to infect fresh Molt-4/8 cells. RT activity appeared in the supernatant 3 days after infection. Peripheral blood lymphocytes were obtained from seronegative AGMs and infected as described above. RT activity was detectable 8 days after infection (data not shown).

Immunoblot analysis of virus derived from the supernatant of transfected cultures is shown in Fig. 3B. In serum from a naturally infected AGM, the pattern of proteins with the cloned virus was indistinguishable from that of the original uncloned virus isolate. The serum reacted predominantly with the viral glycoproteins gp140 and gp45. In contrast, human sera positive for HIV-1 or HIV-2 (Fig. 3B, lanes 4, 5, and 6) showed a strong cross-reactivity with the viral core protein p24. Serum obtained from a pig-tailed monkey (*Macacca nemestrina*) infected with original uncloned SIVagm3 virus reacted with all major viral proteins (Fig. 3B, lane 7).

To test infectivity in vivo, two seronegative juvenile AGMs were inoculated intravenously with 10^5 tissue culture-infectious doses of cell-free virus taken from transfected cultures. Seroconversion was observed within 4 weeks after inoculation. Immunoblot analysis revealed a strong antibody response against all major proteins (Fig. 4, lanes 3, 4, 5, and 6). In contrast, reactivity of sera from naturally infected AGMs was almost exclusively directed against the two envelope glycoproteins of SIVagm (Fig. 3B, lanes 2 and 3).

In an extension of these experiments, one pig-tailed monkey was inoculated with cloned SIVagm3. Antibody responses again occurred within 4 weeks after inoculation, and the pattern of reactivity was very similar to the response of AGMs experimentally infected with cloned virus (Fig. 4, lanes 7 and 8).

Virus could be reisolated from infected AGMs and the pig-tailed monkeys by cocultivation of phytohemagglutinin-stimulated peripheral blood lymphocytes with Molt-4/8 cells. Reisolation was performed 4 and 8 weeks after inoculation. Thus SIVagm3 is biologically active in vitro and in vivo, since infectivity was demonstrated for both AGMs and pig-tailed monkeys. Additional species are presently being tested for susceptibility to SIVagm infection.

DISCUSSION

Wild-type SIVagm virus was isolated from several naturally infected AGMs captured in Ethiopia and kept in our colony at the Paul-Ehrlich-Institut since 1972 (11). In this

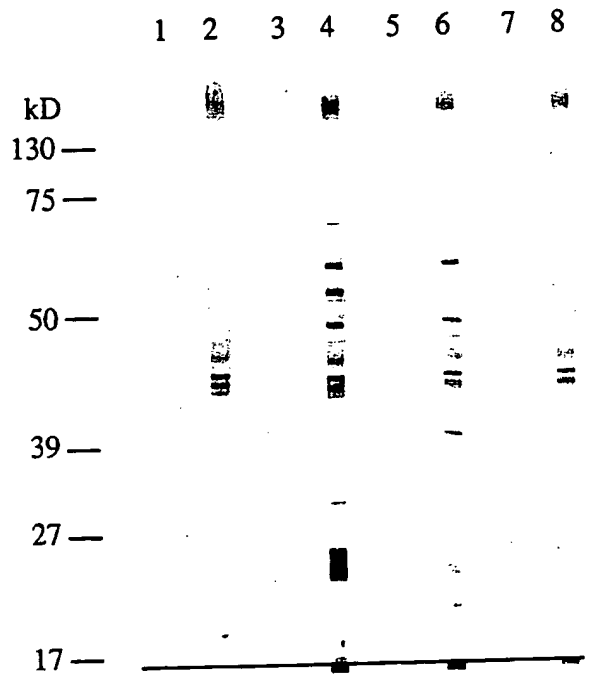


FIG. 4. Seroconversion after inoculation of monkeys with cloned SIVagm3. Lanes: 1, AGM serum negative for SIVagm; 2, AGM serum positive for SIVagm; 3, 5, and 7, prebleedings from two AGMs and one pig-tailed monkey, respectively; 4, 6, and 8, corresponding sera from the same animals 4 weeks after inoculation. kD, Kilodaltons.

study, we describe the molecular and biological characteristics of an infectious molecular clone of SIVagm, termed SIVagm3. A nucleotide sequence comparison was made with the only other published complete nucleotide sequence of a SIVagm isolate (SIVagmTYO-1 from a monkey captured in Kenya). The extent of variability, measured by comparison of restriction maps between our clone SIVagm3 and the published clone SIVagmTYO-1 (Fig. 1), is in the same range as the restriction site divergence found between various HIV-1 isolates (17). Restriction site comparison of our isolate with two other viruses of Kenyan origin, termed SIVagm266 and SIVagm385 (13), revealed a higher degree of divergency than that between SIVagm3 and SIVagmTYO-1. However, more nucleotide sequence data are needed to determine more precisely the variability among individual SIVagm strains.

So far, we have determined the nucleotide sequence of the part of the *env* gene (Fig. 2) that represents the carboxy terminus of the TMP. Clearly, overall homology of 67% with the equivalent region of SIVagmTYO-1 is low compared with the homology of TMPs between various HIV-1 isolates, which has been found to be as high as 85% among otherwise highly divergent members of the HIV-1 family (1).

It is also important at this point to note the absence of a stop codon in the TMP of SIVagm3. A premature stop codon was found in SIVagmTYO-1, which results in the expression of a TMP of 32 kilodaltons rather than 45 kilodaltons. The amino acid homology upstream and downstream of the stop codon present in SIVagmTYO-1 is 75 and 58%, respectively. It is obvious that the homology decreases in the noncoding TMP region downstream of the stop codon, probably because selection for a functional protein region is unnecessary. Intragroup differences of TMP sizes have also been reported for HIV-2 (21) and SIVmac (15) but not for HIV-1. It should be mentioned that other isolates of SIVagm from our colony have been analyzed by immunoblot analysis for the size of their respective TMPs (11) and that they all show a large glycoprotein of 45 kilodaltons, although they are clearly different as determined by restriction site analysis. Other reports have mentioned SIVagm variants with TMP ranging from gp32 to gp38 (10, 16), which suggests that our isolate, with a gp45, is the only SIVagm isolate so far described that lacks the premature stop codon. Exact determination of the functional consequences of TMP size variability awaits further analysis.

SIVagm represents an animal model for infection of a natural primate host with a lentivirus, and further investigations require the biological and molecular characterization of the virus. We were able to isolate a full-length molecular clone of SIVagm that is competent for replication in vitro as well as in vivo. The characteristic syncytium formation observed after infection of CD4-positive T cells with wild-type SIVagm is also a property of our molecular clone SIVagm3. Furthermore, for both wild-type and cloned SIVagm3, chronically infected cultures show a high RT activity in the supernatant with no further indication of cell death (Fig. 3A). In vivo infectivity for juvenile AGMs with SIVagm3 correlated with in vitro infectivity for AGM peripheral blood lymphocytes, as has been previously described for SIVmac (15). It is an important result of our study that pig-tailed monkeys can also be infected with SIVagm. This makes it possible to analyze the properties of SIVagm in primates other than its natural host. It is as yet unknown whether the virus is also apathogenic in heterologous monkey species. Pathogenicity in an unnatural host species might possibly reflect a specific suppression of SIVagm in its natural host. Lack of host factor-mediated suppression in other species would then lead to disease induction. However, AGMs and pig-tailed monkeys, which to date have been infected with the cloned virus for 6 months, show so far no symptoms of disease development.

Interestingly, AGMs and pig-tailed monkeys infected with cultured virus, both native and cloned, developed a strong antibody response to all viral proteins (Fig. 4). In contrast, naturally infected AGMs show reactivity only against major *env* proteins. This difference may reflect a specific suppression of SIV in its natural host after long-term infection or may be due to initial infection with a low titer of virus (presumably via sexual routes) compared with the high titer of infection by experimental inoculation. It may also be due to the fact that juvenile monkeys were used for experimental infection, whereas natural infection only appears to occur in sexually mature animals (data not shown).

The availability of a biologically fully competent molecular clone of SIVagm allows us to now address various pertinent questions in an animal model system which should help to understand features of HIV infection in human beings. It will be crucial to obtain complete sequence information of additional SIVagm isolates. Knowledge of the evolutionary development of the viral anchestry of HIV may well help us to understand the pathogenicity of this virus. As mentioned above, we have recently initiated studies on the extent of variability of SIVagm after infection of monkeys with a single virus clone. This approach may well lead to the determination of the extent and localization of antigenic variation that is characteristic for lentivirus strains. The high extent of variability previously observed might have been due to initial infection with multiple virus substrains and subsequent mutations and recombinations (4). Alternatively,

variation may be a consequence of selection of naturally occurring virus variants during the interplay of viral replication in vivo and immunological pressure by the host. Thus, use of molecularly cloned SIVagm will allow us to verify one of these hypothesis. We will also be able to determine the mutation rate of different viral genes after infection of the natural host and after trans-species transmission.

ACKNOWLEDGMENTS

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MuLV-based vectors pseudotyped with truncated HIV glycoproteins mediate specific gene transfer in CD4+ peripheral blood lymphocytes

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Human immunodeficiency virus (HIV) infection ultimately leads to the destruction of the CD4+ lymphocyte subset and the onset of AIDS. In recent years, several gene therapy procedures making use of retroviral vectors that selectively target HIV susceptible cells have been proposed in order to interfere with HIV productive infection. However, HIV glycoproteins' inability to be incorporated in other heterologous retroviruses considerably limits true HIV cell tropism of such vectors. We now report the use of murine leukemia virus (MuLV) viral particles harboring a truncated form of the HIV glycoprotein for specific gene delivery. Reporter lacZ gene transfer was determined to be appropriately specific to CD4+ cells when HeLaCD4 cells or peripheral blood lymphocytes (PBLs) were infected with these pseudotyped MuLV virus vectors. In contrast, MuLV viruses harboring amphotropic (MuLV) envelope glycoproteins displayed a broad and nonspecific infection of PBL subpopulations. This new approach, taking advantage of the ability of truncated HIV envelope glycoproteins to be incorporated into heterologous retroviral particles, may foreseeably be used in future interventions based on the coordinated delivery of therapeutic gene products specifically to cell types susceptible to HIV infection.

Keywords: *retroviral vector; HIV Env; gene transfer; CD4+ cells*

Introduction

Gene therapy has been proposed as a strategy for the treatment of a variety of genetic and viral diseases. Genetic alteration of the peripheral T lymphocytes has been shown to be of potential therapeutic relevance in inherited diseases, leukemias, and acquired immune deficiency syndrome (AIDS).[1,2] The central goal of many gene therapy strategies is the efficient and stable delivery of genes. Many advantages are associated with the use of well characterized retroviral vectors for this purpose. However, one of the major limitations associated with using retroviral vectors in gene therapy is their low transducing efficiency. Accordingly, several approaches have been developed to increase retroviral transduction efficiency. Methods include optimization of infection conditions, increasing virus titer, and improving the specificity of the virus for its target cells. In fact, the use of nontargeted retroviral vectors in gene therapy is often considered inadequate since binding to nontarget cells would result in possible side-effects in undesired transduced cells. The use of *in vitro* transfer protocols, where cells are cultured and transduced *ex vivo*, can allow partial bypass of these limitations, but restricts the spectrum of diseases which may be treated. In many cases, systematic treatment can only be achieved through the use of retroviral vectors that will deliver genes to specific cells *in vivo* and enable efficient expression in these cells.

Targeting retroviral vectors to specific cells can be achieved through inducible transcriptional control or preferably by achieving a specific infection of the desired cells by targeted transduction. The latter approach requires modification of the proteins involved in receptor recognition. Retroviruses use a variety of receptors to enter cells; some of these receptors such as the ecotropic and amphotropic murine leukemia virus (MuLV) receptors and many of the immunodeficiency-causing lentivirus receptors have been identified.[3–8] However, targeted delivery of a murine-based retroviral vector to a specific subpopulation of human cells has yet to be truly achieved. Initial attempts to change viral tropism were done through the use of pseudotyped viruses.[9–11] MuLV is able to incorporate vesicular stomatitis virus G glycoprotein (VSV-G), influenza virus hemagglutinin (HA), or heterologous retroviral glycoproteins, although this expands viral host range rather than restrict cell tropism. Even though several groups have generated chimeric Env-ligand fusion proteins designed to bind on to transferrin receptor, galactose receptor or the high density lipoprotein receptor, none of these fusion proteins were successfully incorporated into infectious viral particles.[11] The only reported success has been obtained using heregulin Env fusion proteins binding to the Erb3/4-receptor, thus enabling specific MuLV infection of human breast cancer cell lines.[12] An alternative approach using receptor-single chain antibodies (scFv) fused to Env proteins has given rise to several difficulties; results obtained with low density lipoprotein scFv-Env chimeric proteins are still

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promising, although virus titers remain poor.[11,13] Most obstacles found in these strategies arise in the Env post-binding or membrane fusion steps, giving rise to abortive infection.[11] Therefore, whenever possible, it will be preferable to use authentic viral envelope glycoproteins exhibiting specificity towards the desired targeted cells.

Targeted transduction of CD4+ lymphocytes is critical for HIV gene therapy approaches. Although the establishment of HIV-based vectors for such a strategy has been considered, it is more likely that grafting specific components of HIV on to other retroviruses would generate safer vectors. Although HIV's ability to infect nondividing cells is a great advantage over classical retroviral vectors, HIV's specific, targeted tropism for CD4+ cells must also be considered.[1,14,15] Recently, chimeric HIV Env glycoproteins encompassing the cytoplasmic domain of the VSV G-glycoprotein were shown specifically to target VSV infection to CD4 expressing human epithelial HeLa cells.[16] The incorporation of foreign glycoproteins in enveloped viruses, or pseudotyping, is a well known phenomenon. Reported HIV pseudotypes include HIV particles harboring MuLV Env, human T cell leukemia virus (HTLV) Env, VSV-G and herpes simplex virus (HSV) Env.[17-23] However, the inability of the HIV glycoproteins to be incorporated into MuLV viral particles has kept these envelope glycoproteins from being of potential use in gene therapy. The presence of a 150-amino acid long intracytoplasmic tail in the HIV gp41 transmembrane glycoprotein is believed to be the cause of such restricted incorporation of HIV Env; steric incumbrance and structure incompatibility between the long gp41 cytoplasmic domain and non-HIV matrix or capsid proteins would explain the specific incorporation.[24] This was further confirmed when HIV Gag matrix protein mutations were shown to disrupt the incorporation of HIV Env.[25-27] Although the incorporation of HIV Env into the viral particle needs a specific interaction with the HIV matrix protein, other retroviral Env glycoproteins seem to bypass this step.[28,29] This correlates well with the much shorter cytoplasmic domains of most retroviral Env glycoproteins.

We now confirm and extend recent observations by Mammano *et al*[30] and Schnierle *et al*,[31] showing that truncation of the cytoplasmic portion of the Env glycoprotein enables its incorporation into heterologous MuLV virions. Furthermore, truncated envelope glycoproteins were efficiently incorporated into HIV virions harboring matrix protein mutations known to affect glycoprotein incorporation. MuLVs harboring the truncated HIV glycoproteins retain normal biological activity and specifically infect HeLa CD4 expressing cells and activated CD4+ peripheral blood lymphocytes (PBLs), as determined by transduction of a *lacZ* reporter gene. Altogether, these results demonstrate the feasibility of using this approach to specifically transduce genes into the CD4 expressing cell subpopulation for gene therapy purposes.

Results

Incorporation of full-length and truncated HIV Env glycoproteins into viral particles

In order to investigate the incorporation of envelope glycoproteins, wild-type HIV Env expression vectors were cotransfected into 293T cells with either HIV Env-negative proviral DNA (HIVGag) or a MuLV GagPol-encoding plasmid (MuLVGag, described in Figure 1). After a 36 h delay following transfection, cells were subjected to metabolic radiolabeling with ^{35}S methionine, as described in Materials and methods. Viral proteins were then immunoprecipitated with HIV-positive patient and goat anti-MuLV Gag antisera and analyzed by SDS-PAGE. Cells transfected with the HIV Env-negative DNA or MuLV GagPol encoding plasmid alone expressed, respectively, the HIV or MuLV major Gag viral capsid proteins, p24 (HIV) or p30 (MuLV) (Figure 2a, lanes 2 and 3). These proteins were also detected in the virus particles recovered by ultracentrifugation of cell supernatants followed by immunoprecipitation (Figure 2b, lanes 2 and 3). When cells were cotransfected with the HIV Env expression vector and those encoding either HIV or MuLV Gag, the HIV glycoprotein gp120 could also be detected in both cell lysates (Figure 2a, lanes 4 and 5). However, incorporation of the HIV Env was restricted to the HIV viral particles (Figure 2b, compare lanes 4 and 5).

If the length of the HIV Env cytoplasmic domain is responsible for such a specific virion incorporation, full truncation of this domain should abrogate this restriction to virion incorporation and should confer to truncated HIV glycoproteins broad pseudotyping abilities, comparable with those of MuLV or other retroviral glycoproteins. To look into such a possibility, cells were again cotransfected with either the proviral HIV Env-negative plasmid or the MuLV GagPol encoding plasmid, and an expression vector encoding a HIV Env glycoprotein deleted of its carboxy-terminal 144-amino acids (HIVEnvΔ713-856, Figure 1b). Analysis of radioimmunoprecipitated proteins shows that the HIV Env truncated glycoproteins are correctly processed (Figure 2a, lanes 6 and 7) and incorporated into both HIV and MuLV virions (Figure 2b, lanes 6 and 7). This establishes that the specific incorporation of the HIV Env glycoproteins into the HIV viral particle, and the exclusion of such glycoproteins from other enveloped viruses thereof, is dependent on the presence of the HIV Env long cytoplasmic domain.

To investigate further the role of the HIV Env cytoplasmic domain in its specific incorporation into the HIV viral particle, an HIV Env-negative proviral DNA harboring a small deletion (amino acids 16-18) in the HIV matrix protein was generated (Figure 1b). This small deletion has been previously identified as abrogating HIV Env virion incorporation.[25,32] This proviral DNA construct was cotransfected into cells with either the wild-type HIV Env, truncated HIV Env or MuLV Env glycoprotein expression vectors. Although the major capsid proteins (MuLV p30 and HIV p24) and all the different Env glycoproteins (MuLV gp70 and HIV gp120) could be detected in the cell lysates (Figure 2a, lanes 8, 9 and 12), the full-length HIV Env glycoproteins were specifically excluded from the p17-mutant virions (Figure 2b, lane 8), as was also the case in the MuLV virions (Figure 2b, lane 5). In contrast, the truncated HIV Env was incorporated in the p17 mutant virion (Figure 2b, lane 9). The MuLV Env, with its short intracytoplasmic domain, was uniformly incorporated into wild-type or mutant HIV virions or the MuLV virions (Figure 2b, lanes 11, 12 and 13). Altogether, these results demonstrate the role of the HIV Env cytoplasmic domain in specific incorporation into HIV, through a possible

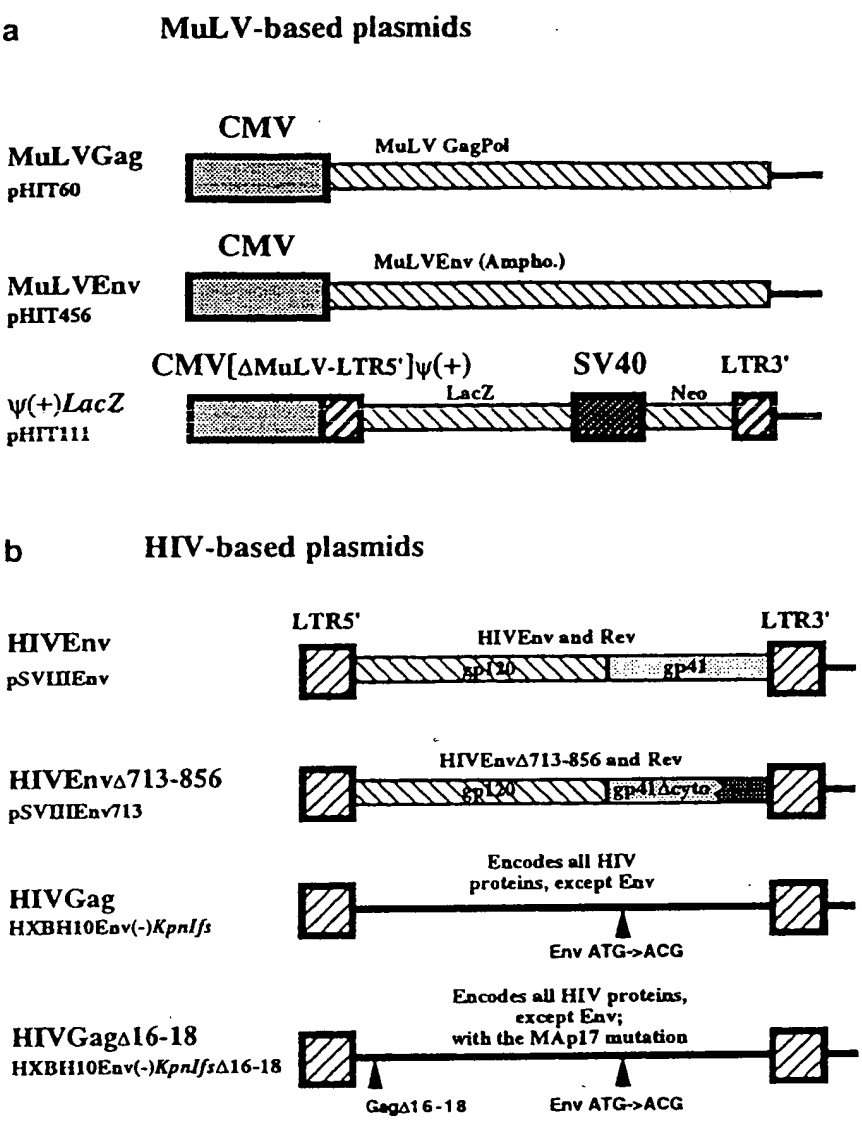


Figure 1 Schematic representation of plasmids and expression vectors. (a) MuLV-based plasmids. All plasmids contain an SV40 origin of replication, and transcription is driven by a CMV promoter.[36] pHIT111 is peculiar in that the CMV promoter is fused to the R region of the 5' MuLV LTR. This plasmid also harbors a neo resistance gene under transcriptional control of the SV40 promoter and 3' MuLV LTR sequences. (b) HIV-based plasmids. Transcription is driven by the HIV LTR, and therefore needs the presence of Tat for efficient expression of the reporter gene, and the SVCMVTat plasmid is thus used to supply Tat in experiments involving the HIV Env encoding plasmids and MuLVGag. All plasmids contain an SV40 origin of replication. The Env-negative proviral HIV plasmids HXBH10Env(-)KpnIfs[37] harbor an ATC to ACG point mutation at the initiation codon of Env and a KpnI frameshift at HIV-HxBc2 nucleotide position 5934. A stop codon at position 7902 is inserted in the HIV truncated-Env glycoprotein expression vector HIVEnvΔ713–856, resulting in the deletion of the cytoplasmic domain of the glycoproteins.[30] A small deletion in the p17-matrix encoding portion of the gag gene in HXBH10Env-(KpnIfs) Δ16–18 abrogates wild-type Env viral incorporation.

interaction with the HIV p17-matrix protein. The deletion of such a cytoplasmic domain eliminates any need for such a specific interaction, thereby rendering possible incorporation into MuLV virions.

Infectivity of pseudotyped MuLV virions

Incorporation of the truncated HIV Env glycoproteins into the MuLV viral particle being established, it was then necessary to determine if the pseudotyped MuLV viruses had incorporated fully functional envelope glycoproteins giving rise to infectious viruses. If such is the case, the viruses should specifically infect $CD4^+$ cells. Either the HIV Env-negative proviral DNA, the HIV p17-matrix mutant Env-negative proviral DNA or the MuLVGag expression vector were thus cotransfected in COS cells with the HIV Env, truncated HIV Env, or MuLV Env encoding vectors, as described in Materials and methods. Cotransfections using MuLVGag vector also included a plasmid harboring a *lacZ* gene and RNA viral encapsidation sequences (Figure 1a). Such encapsidation sequences being absent on MuLVEnv or MuLVGag, *lacZ* RNA is thus exclusively incorporated into MuLV virions using this approach. Conversely, the HIV p17-mutant or wild-type Env-negative full-length RNAs are encapsidated into their respective viral particles in all experiments involving HIV. Cell supernatants were harvested 48 h after transfection, and relative amounts of recovered virus were estimated by reverse transcriptase

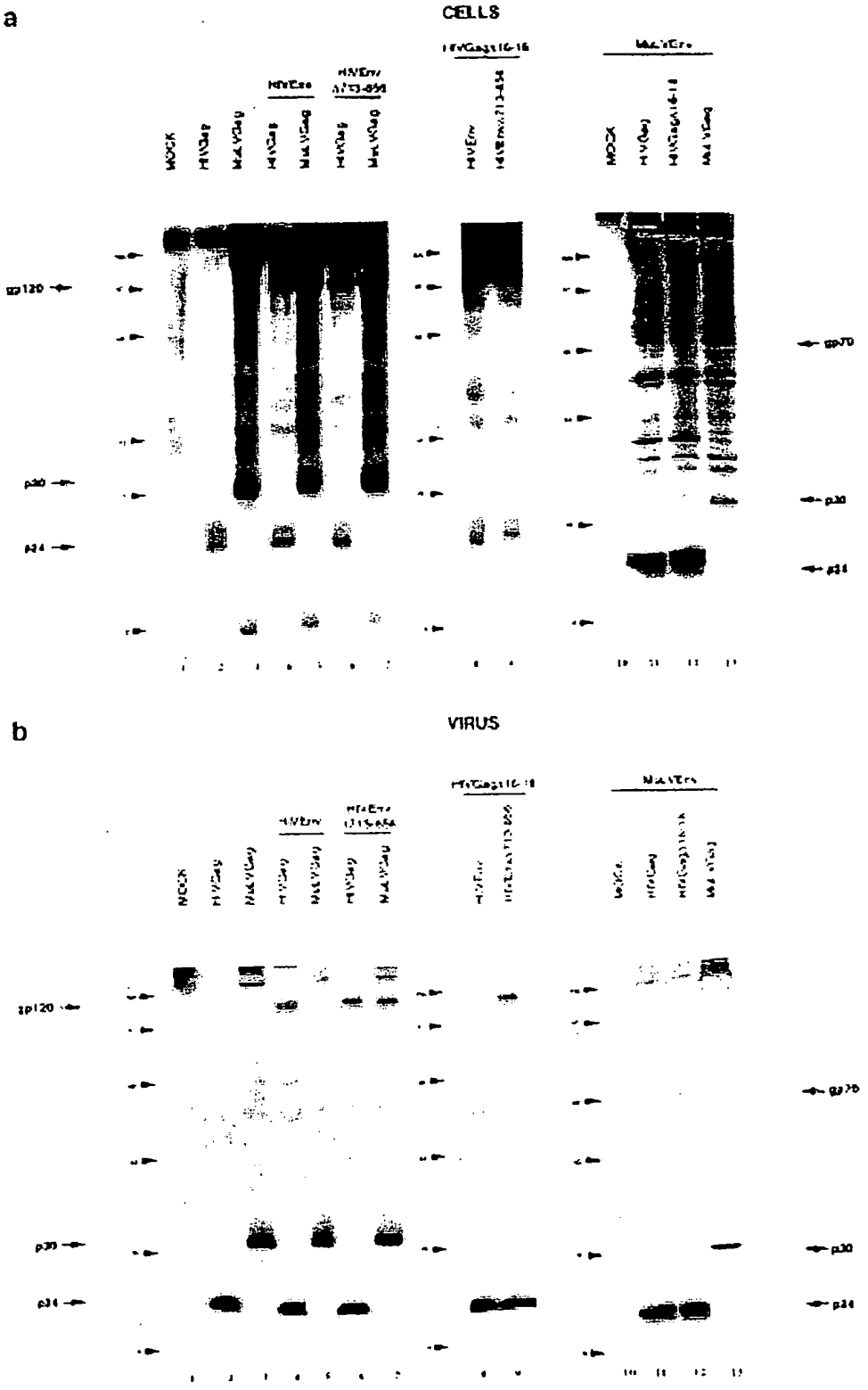


Figure 2 Expression and assembly of MuLV and HIV viral proteins. (a) Cells were cotransfected with the relevant Gag and Env encoding plasmids. Viral proteins were immunoprecipitated with an HIV-positive human serum combined with goat anti-MuLV Gag and anti-MuLV Env antisera, and resolved on an 11% SDS-PAGE gel. (b) Incorporation of glycoproteins in released HIV or MuLV virions. Labeled viral pellets were lysed in RIPA buffer, and immunoprecipitated as in (a) and viral proteins resolved on an 11% SDS-PAGE gel. Labeled proteins were then revealed by autoradiography Position of molecular weight markers is shown and viral proteins are indicated by arrows.

(RT) assay. Equivalent amounts of virus produced from each cotransfection were used to infect either HeLa, HeLaCD4 or HeLaCD4LTRβ-gal cells. Following infection, cells were fixed and stained as described in Materials and methods.

As expected, in HeLa cells, only MuLV-based viruses harboring the amphotropic MuLV envelope glycoprotein were able to transduce the *lacZ* gene (Figure 3). Since HeLa cells lack the CD4 receptor, all viruses harboring HIV glycoproteins did not infect the cells. Moreover, the HIV-based viruses used in this study do not possess intrinsic *lacZ* transduction ability, and therefore do not provide further information when used on these cells (Figure 3b).

In HeLaCD4 cells, similar transduction efficiencies were obtained with MuLV-based viruses having incorporated either the amphotropic MuLV Env or the truncated HIV Env glycoproteins (Figure 3a). Use of all other viruses resulted in background β-galactosidase activity (Figure 3). Again, no detection of HIV-based viruses was expected using these cells. The susceptibility of HeLaCD4 cells to the MuLV viruses harboring the truncated HIV Env demonstrates the functional capacity of such an envelope glycoprotein to mediate specific infection of CD4+ cells and confirms the lack of incorporation of wild-type HIV Env in MuLV. Furthermore, the infectious potential of viruses harboring amphotropic Env or truncated HIV Env appears to be similar.

Finally, virus infection was tested on HeLaCD4LTRβ-gal cells. These cells harbor in their genome a *lacZ* gene under the transcriptional control of the HIV LTR promoter. Following HIV infection, and production of the Tat transactivating protein, they will thus express β-galactosidase activity due to Tat-mediated LTR transactivation.[33] In these cells, MuLV-mediated *lacZ* transduction was obtained when using the HIV truncated-Env or amphotropic MuLV Env glycoproteins, as in the HeLaCD4 cells (Figure 3a). Furthermore, HIVs having incorporated wild-type or truncated Env glycoproteins also efficiently gave rise to β-galactosidase activity in the cells, through Tat transactivation of endogenous *lacZ* (Figure 3b). However, mutant HIVs harboring the mutation in p17 known to prevent incorporation of HIV Env glycoproteins, remained uninfectious in the case of the wild-type HIV glycoproteins only (Figure 3c). Similar efficiency of infection was obtained when the truncated HIV Env glycoproteins were incorporated into HIV, HIV (p17-mutant) or MuLV-based viruses. Altogether, these results demonstrate that the deletion of the HIV Env glycoprotein cytoplasmic domain does not alter the processing, maturation and ability of such glycoproteins to confer an infectious potential for CD4+ cultured cell lines.

Reporter lacZ gene transduction in activated PBLs

Since the truncated HIV Env glycoproteins enable MuLVs to infect CD4+ HeLa cells specifically, it should be possible to take advantage of such a mechanism to infect primary human cells expressing the CD4 receptor. MuLVs harboring either HIV truncated Env or amphotropic MuLV Env glycoproteins were added to PHA-acti-

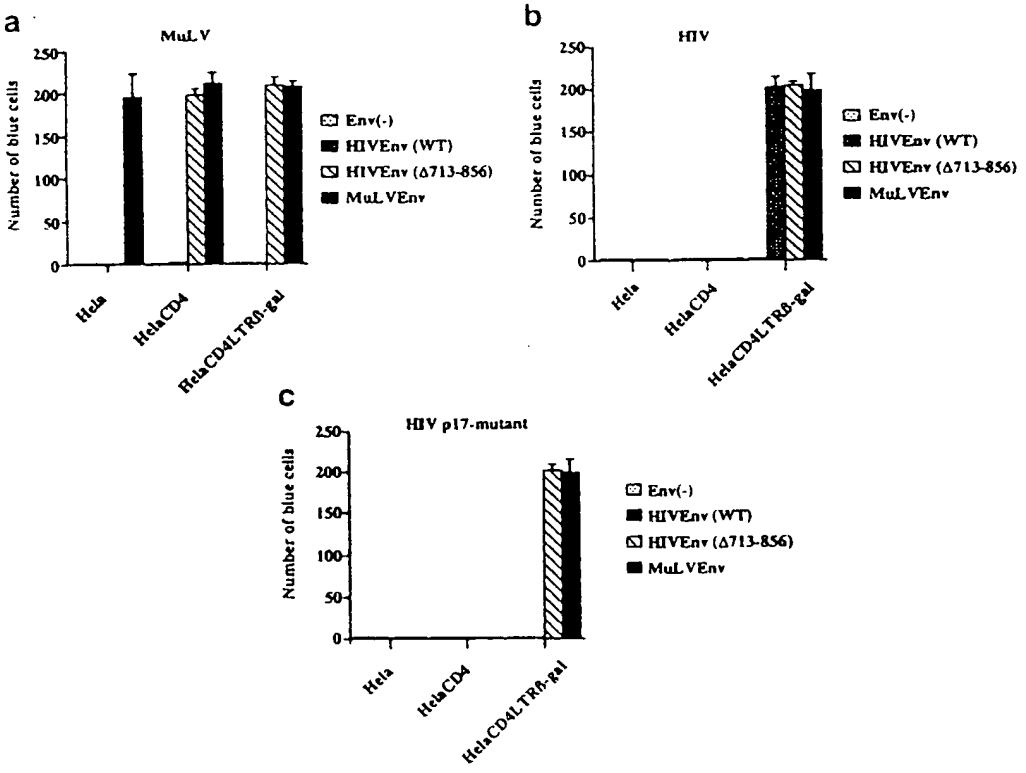


Figure 3 Infection with MuLV (a), HIV (b) or HIV p17-mutant (c)-based viruses harboring either MuLV Env, HIV Env, HIV truncated Env or no Env glycoproteins. Viruses were produced in COS cells, and volumes corresponding to equivalent virus amounts according to reverse transcriptase values were used to infect either one of the HeLa, HeLaCD4 or HeLaCD4LTRβ-gal cell lines, as described in Materials and methods. Cells were then stained with X gal and infected cells identified by the development of a blue color. Results are the average of two experiments and error bars represent standard deviation.

vated PBLs, as described in Materials and methods. MuLVs originating from cells expressing either wild-type HIV Env or no glycoproteins were used as negative controls, since the full-length HIV Env is not incorporated into MuLV. The same experiment was performed in the presence of antibodies against HIV Env (anti-gp120).

Following a delay to allow sufficient β-galactosidase expression, cells were fixed and stained for β-galactosidase expression. However, sensitivity of this assay was poor. PCR Southern analysis was therefore used to detect transduced *lacZ* DNA in infected cells. PCR was used to generate a fragment of 480 bp of the *lacZ* gene, and the nature of the reaction product was confirmed by Southern blot using a specific ^{32}P-labeled *lacZ* probe. The detection of *lacZ* DNA in cells infected with the MuLV viruses having incorporated either truncated HIV Env or MuLV amphotropic Env was easily achieved. Furthermore, *lacZ* gene transfer was specifically prevented upon treatment with anti-gp120 in the case of the truncated HIV Env glycoprotein MuLV pseudotype (Figure 4a). Amplification of the control GADPH gene confirmed the quality of each DNA sample. These results demonstrate the targeted delivery of a transduced *lacZ* gene into activated PBLs through the use of a MuLV retroviral vector harboring heterologous truncated HIV envelope glycoproteins. Identical results were obtained when cells were collected from a second blood donor.

Specificity of infection in PBLs

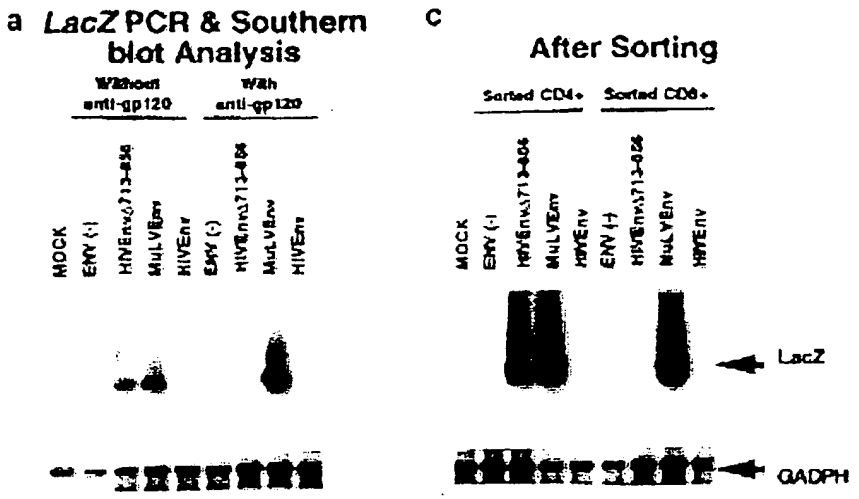
Although the specific infection of the CD4$^+$ cell population was suggested by the inhibitory effect of antibodies against gp120, CD4/CD8 cell sorting was further performed in order to identify the activated PBL populations targeted by the pseudotyped MuLVs. Activated PBLs were infected as described previously and sorted into separate CD4 expressing and CD8 expressing PBL subpopulations. A typical distribution of a sorted infected cell population is shown in Figure 4b. Subpopulations of CD4$^+$ or CD8$^+$ cells were then lysed and PCR Southern blot analysis performed to determine which subpopulation supported *lacZ* transduction. Both CD4$^+$ and CD8$^+$ PBL subpopulations acquired *lacZ* DNA upon infection with the MuLV virus harboring amphotropic Env (Figure 4c). However, only the CD4$^+$ cells demonstrated susceptibility to MuLVs harboring truncated HIV Env (Figure 4c). These results demonstrated the capacity of pseudotyped MuLVs to target and specifically infect CD4$^+$ PBLs.

The efficiency of *lacZ* transduction in sorted CD4$^+$ PBL subpopulations was estimated by Southern blotting of fragments obtained by PCR of serial dilutions of DNA from cells transduced with either MuLV Env or HIV Env harboring viruses (Figure 5). *LacZ* detection by this procedure in cells transduced with the HIV Env viruses required an approximately four-fold higher amount of DNA than the detection in cells infected with the amphotropic virus (compare lanes 2-7). Moreover, control cellular GADPH single-copy DNA could be detected at DNA levels similar to that of transduced *lacZ* in cells infected with the amphotropic virus. Since controls for *lacZ* and GADPH PCRs reveal similar efficiency in serial dilutions of plasmids harboring the respective genes (results not shown), this suggests that transduction efficiency with the amphotropic virus is close to 100%. Therefore, despite the fact that infection was performed under conditions optimized for MuLV Env, truncated HIV Env viruses transduced *lacZ* efficiently, approaching 20-25% of the CD4$^+$ cells.

Discussion

In the present study, it was demonstrated that an HIV Env in which the 144 carboxy-terminal amino acids of the cytoplasmic domain have been deleted can still be incorporated into wild-type HIV particles. This contrasts with the situation observed with smaller deletions showing altered incorporation into the virion.[34] These observations can be reconciled, however, since the HIV Env with a complete truncation of its intracytoplasmic domain is also incorporated into HIV viruses harboring known matrix mutations that normally interfere with HIV Env incorporation.[27,28] Truncation of the cytoplasmic HIV Env domain apparently alleviates any need for specific HIV Gag interaction, effectively converting the incorporation mechanisms to that of shorter cytoplasmic-domain glycoproteins. Accordingly, the truncated glycoprotein was incorporated into MuLV viral particles, in contrast to full-length envelope. Similar observations were also reported by other groups.[30,31] Nevertheless, in order for the truncated glycoprotein to be useful for retroviral vectors, it must retain the general features of the wild-type glycoprotein, namely, correct processing, CD4$^+$ interaction and membrane fusion, all prerequisites to the release of the nucleocapsid into the cytoplasm. In fact, it proved to be, that in CD4 expressing HeLa cells, the infectious potential of the pseudotyped MuLV viruses was comparable to that of MuLV viruses harboring amphotropic MuLV Env, or HIV viruses possessing either full-length or truncated forms of HIV Env, as previously reported.[30] Moreover, pseudotyped viruses exhibited similar infectivity without the additional presence of full-length MuLV or HIV Env, as was shown necessary in the case of chimeric ligand-Env glycoproteins.[35] Finally, specific, targeted infection of the CD4$^+$ PBL subpopulation was obtained using the MuLV-based vector pseudotyped with the truncated HIV Env. Interestingly, despite the fact that infection was performed under conditions optimized for MuLV Env, truncated HIV Env viruses transduced PBLs with only slightly inferior efficiency to that of amphotropic MuLV viruses. These modified viral vectors are thus able to recognize efficiently and specifically infect CD4$^+$ cells in a mixed cell population.

There could be numerous advantages in using such a vector in gene therapy for AIDS. The true tropism of HIV, including possible CD4-independent cell tropism, should be retained by such pseudotyped viruses. Furthermore, tropism to CD4$^+$ macrophages or lymphocytes could be achieved by pseudotyping MuLV with truncated macrophage-tropic or T-tropic HIV Env glycoproteins. Access to CD4$^+$ nondividing cells (such as dendritic cells and macrophages) could eventually be obtained by grafting other functional elements of the HIV virus, such as the HIV matrix or Vpr proteins, on to MuLV viral vectors coated with such HIV truncated Env glycoproteins. This strategy could retain desired functions of the HIV virus while alleviating the potential of HIV replication through recombination events, or even synthesis of most HIV proteins, in gene therapy treatments. These CD4$^+$ cell-targeted MuLV vectors strategies are to be developed with



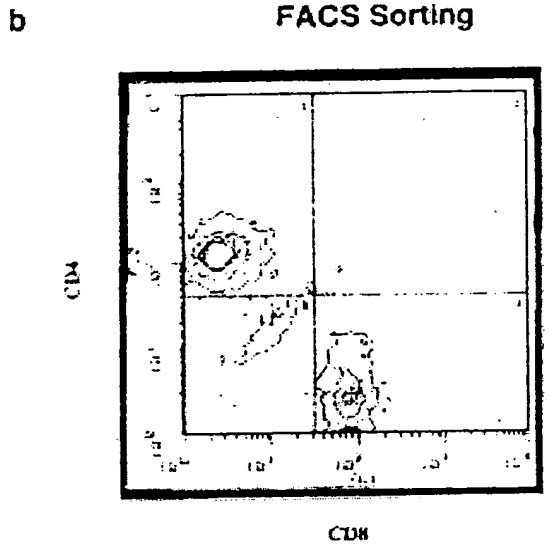


Figure 4 (a) LacZ transduction of PBLs as determined by PCR amplification of lacZ and Southern analysis. MuLV viruses harboring various glycoproteins were produced in COS cells and used to infect PBLs as described in Materials and methods, with or without concomitant addition of anti-gp120. Cells were then lysed and genomic DNA extracted. PCR was performed on aliquots of extracted DNA, followed by Southern blotting and hybridization with a lacZ specific probe. (b) Representative distribution of CD4 and CD8 populations of infected cells as determined by FACS analysis. Aliquots ($2-3 \times 10^6$) of infected cells were treated with a mix of anti-CD8 fluorescein isothiocyanate and anti-CD4 phycoerythrin-conjugated antibodies and sorted with a FACSTAR cell sorter. (c) Southern analysis of transduced cells following sorting. Infected cells were sorted as CD4+ or CD8+ cells, and transduction of lacZ was determined by PCR amplification and Southern analysis as in (a). Amplification of a GADPH gene fragment was performed in parallel on each DNA sample and the results are presented at the bottom of (a) and (c).

the goal of delivering inhibitors of HIV replication in specific cells. Such viral targets could include either early or late steps in the viral replicative cycle. Therapeutic strategies include transduction of genes inhibiting proviral integration or gene expression, or activating suicide genes in HIV-infected cells. The use of gene therapy in AIDS could therefore focus on poorly accessible HIV susceptible cells, which harbor latent viruses, that remain the origin of viral resistance encountered within the current antiretroviral therapies. Finally, the system developed in the present report is of great relevance for the study of gene expression in specific CD4+ cell populations.

Materials and methods

Proviral constructs and expression plasmids

Plasmids pHIT60 and pHIT456 respectively encode the GagPol protein of MuLV and the amphotropic Env glycoprotein of MuLV. The pHIT111 plasmid harbors a *lacZ* gene under the transcriptional control of a modified CMV promoter that is fused to the R-U3 regions of the 5′ MuLV LTR and Ψ-encapsidation sequences. All the pHIT plasmids were obtained from AJ Kingsman (Oxford University, Oxford, UK).[36] Plasmid pSVIIIEnv713 was created by cloning the *Kpn*I–*Bam*HI (corresponding to

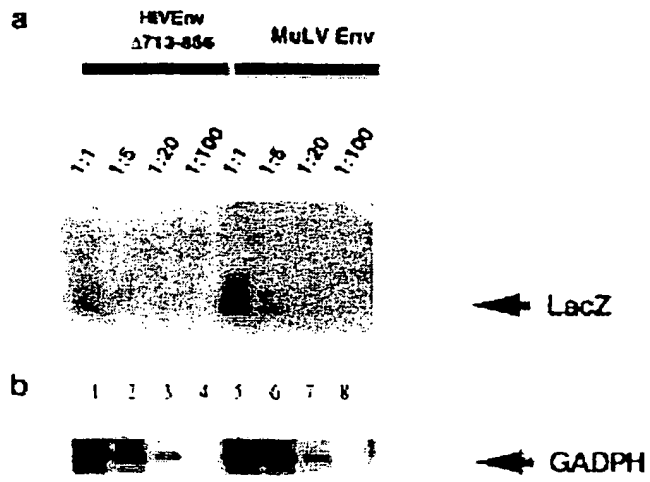


Figure 5 (a) LacZ transduction efficiency of truncated HIV Env and amphotropic Env MuLV viruses. Efficiency of lacZ transduction in sorted CD4+ PBL subpopulations was determined by Southern blotting of fragments obtained by PCR of shown serial dilutions of DNA from cells transduced with either MuLV Env or HIV Env harboring viruses. Control cellular GADPH DNA is shown in (b).

nucleotide positions 5893 and 8017 of HIV HXBc2 proviral DNA, respectively; +1 = site of initiation of transcription) segment of HXBH10ΔCT,[30] a proviral DNA containing a stop codon in *env* (corresponding to a TCA to TAA substitution at HIV-HxBc2 nucleotide position 7902), and encoding an Env truncated of its cytoplasmic domain (obtained from Heinrich G Gottlinger, DFCI, Boston, MA, USA) in pSVIIIEnv, the vector used for expression of Env under the control of the HIV LTR promoter. The Env-negative proviral construct harboring a mutation in the matrix protein HXBH10Env-(*KpnIfs*)Δ16-18 was generated by cloning the *SalI-BamHI* (HXBH10 nucleotide positions 5372 and 8058, respectively; +1 = site of initiation of transcription) fragment of HXBH10Env-(*KpnIfs*) into HXBH10Δ16-18; both plasmids have been described elsewhere.[25,37,38]

Cell lines and antisera

The 293T, COS, HeLa, HeLaCD4[39] and HeLaCD4LTRβ-gal[33] cells were maintained in DMEM supplemented with 5% fetal calf serum (FCS) and 1% streptomycin and penicillin. Human antiserum No. 162 against HIV proteins has been described elsewhere.[37] The goat antiserum against MuLV Gag (76S000127 and 79S000804) was obtained from Quality Biotech (Biological Carcinogenesis Branch, NCI, Camden, NJ, USA); antiserum raised against MuLV Env (80S00019) was also obtained from Quality Biotech.

Transfection, metabolic radiolabeling and radioimmunoprecipitation

DNA transfection in 293T cells (10^6) was performed using liposomes, as previously described.[37] Briefly, 10 μg of the GagPol encoding plasmids (pHIT60, HXBH10Env-(*KpnIfs*) or HXBH10Env-(*KpnIfs*)Δ16-18) were cotransfected with 15 μg of one of the following Env encoding plasmid constructs: pHIT456, pSVIIIEnv or pSVIIIEnv713. When pHIT60 was cotransfected with the HIV Env or truncated HIV Env plasmid expression vectors, 5 μg of a HIV transactivating protein (Tat) encoding plasmid (SVCMVTat) was added to the DNA mix before transfection, since transcription of both Env genes is driven by the HIV LTR, and their efficient expression is thus dependent on the presence of Tat. Following a 36 h transient expression period, cells were labeled with 150 μCi/ml of ^{35}S methionine (Tran^{35}S-label; ICN Biomedicals, Irvine, CA, USA) for 8 h in methionine free-medium. Supernatants were harvested and ultracentrifuged (100 000 *g* for 1 h in a Beckman Ti50.4 rotor; Beckman Instruments, Mississauga, Ontario, Canada) to generate a crude viral pellet; cells and viral pellets were then resuspended in RIPA lysis buffer (10 mM Tris-HCl (pH 7.4), 1mM EDTA, 100 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.25% sodium deoxycholate, 0.2% phenylmethylsulfonyl fluoride (PMSF)). Viral proteins present in lysed cells or viral pellets were immunoprecipitated with a mix consisting of HIV-positive human, goat anti-MuLV Gag and goat anti-MuLV Env antisera (all used at 1:5000 dilutions) and loaded on to an 11% SDS-PAGE gel as described previously.[37] Labeled proteins were then revealed by autoradiography.

Infectivity assay using β-galactosidase activity

The infectivity assay used to determine if viruses harboring the different Env glycoproteins retained infectious capacity was similar to the one described by Kimpton and Emerman,[33] with several modifications, in order to comply with the MuLV *lacZ* transduction strategy elaborated by Soneoka *et al*.[36] Briefly, COS cells were lipofected with various combinations of GagPol encoding DNA and Env expression vectors as described in the previous section. Furthermore, 15 μg of the pHIT111 *lacZ* vector was added to every transfection when pHIT60 was present, in order to obtain *lacZ* transduction through MuLV viruses. At 40 h after transfection, viruses were harvested in 2 ml of DMEM supplemented with 8% FCS and filtered on 0.45 mm pore diameter filters (Millipore, Bedford, MA, USA) to remove cellular debris. A reverse-transcriptase assay was performed on aliquots of the filtered supernatants[40,41] to determine virus yield. Equivalent amounts of the HIV (100 000 c.p.m.) or MuLV (150 000 c.p.m.) viruses were used to infect either HeLa, HeLaCD4 or HeLaCD4LTRβ-gal cells, seeded the previous day in 24-well plates at 3×10^4 cells per well. Polybrene (10 μg/ml) was added to the infected cells and diluted 8 h into the infection with fresh DMEM supplemented with 8% FCS and antibiotics. Finally, cells were washed, fixed and stained as previously described[33] 40 h after the start of infection and the number of blue cells determined using light microscopy.

Culture, isolation and infection of PBLs

Peripheral blood mononuclear cells (PBMCs) were obtained from volunteers by Ficoll-Paque centrifugation as recommended by the manufacturer (Pharmacia, Baie d'Urfe, Quebec, Canada) and washed thoroughly to remove platelets. PBLs were enriched from the PBMCs by two-step adherence to remove contaminating macrophages. Cells were cultured in the presence of phytohemagglutinin (PHA, 5 μg/ml) for 72 h to obtain activated lymphocytes. Following activation, cells were washed several times to remove the lectin and were maintained in RPMI supplemented with glutamine and 10%

FCS, penicillin-streptomycin (100 U/ml), gentamycin (10 μg/ml) and 20 U/ml interleukin-2 (rIL2; Boehringer Mannheim, Laval, Quebec, Canada) at 37°C in a 5% CO_2 incubator. Infection of PBLs was performed in conditions similar to those described by Bunnell *et al*.[42] Briefly, 1×10^6 PBLs were seeded in six-well plates, at 2 ml per well, in phosphate-free RPMI supplemented with 10% FCS and rIL2 for 8 h, since phosphate depletion has been shown to enhance MuLV receptor expression in susceptible cells. Subsequent to the phosphate depletion step, cells were exposed to viruses (at a MOI of 0.1 as determined by the number of blue cells obtained with HeLaCD4LTRβ-gal cells) and centrifuged at low speed and 32°C for 60 min in a Beckmann GS-6R centrifuge (Beckman Instruments). Following centrifugation, cells were incubated for 12 h at 32°C, washed and maintained in RPMI supplemented with 10% FCS, rIL2 and the antibiotics as above. A second series of infected cells was treated with sheep anti-gp120 at a dilution of 1:250 throughout the infection procedure in order to determine the specificity of the viruses for targeting the $CD4^+$ cell population.

Analysis of infected PBLs by PCR and Southern blot

A *lacZ* PCR Southern blot strategy was used to detect transduced *lacZ* DNA in PBLs. Briefly, cells were lysed by repeated freezing and thawing, cell nuclei pelleted and treated with proteinase K (0.1 mg/ml) 1% SDS in Tris-buffered saline for 4 h at 50°C. DNA was extracted by two steps of phenol-chloroform and ethanol precipitation. A fifth of this crude extract was used to detect *lacZ* gene transduction by PCR amplification. Amplification was carried out using *Taq* DNA polymerase (Perkin Elmer, Norwalk, CT, USA) and required 30 cycles of 1 min at 94°C; 2 min at 50°C and 3 min at 72°C. The primers used to amplify *lacZ* were 5'-CTTCTATAGA CACGCAAACAC-3' (sense) and 5'-CCCGTCGGA TTCTCCGTCGG-3' (antisense). Detection of the *lacZ* sequence was performed by Southern blotting and hybridization with a probe corresponding to the homologous *lacZ* region and generated by nick-translation using α^{32}P-dATP (3000 Ci/mmol; ICN Radiochemicals, Irvine, CA, USA). The glyceraldehyde-3 phosphate dehydrogenase (GADPH) primers that were used as internal PCR amplification controls were 5-GCTCATGCCCCCATG TTCCT-3 (sense oligonucleotide) and 5-CAAAGG TGGAGGAGTGGGTGT-3 (antisense oligonucleotide). The GADPH amplified product was detected using a probe covering the entire GADPH sequence[43] and generated by nick-translation.

FACS analysis of the transduced cells was performed with Cyto-Stat/Coulter Clone T8-FITC anti-CD8 fluorescein isothiocyanate or T4-RD1 anti-CD4 phycoerythrin-conjugated antibodies (Coulter Corporation, Miami, FL, USA). Following extensive washing, approximately $1-2 \times 10^5$ $CD4^+$ or $CD8^+$ cells were sorted using a FACSTAR cell sorter (Becton Dickinson, Mountain View, CA, USA). Sorted cells were lysed and analyzed for *lacZ* transduction by PCR and Southern blot analysis as previously described. Quantification of signals on autoradiograms was performed with a Molecular Dynamics Personal Densitometer (Sunnyvale, CA, USA) using ImageQuant software.

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MLV-Derived Retroviral Vectors Selective for CD4-Expressing Cells and Resistant to Neutralization by Sera from HIV-Infected Patients

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Retroviral vectors derived from amphotropic murine leukemia viruses (MLV) mediate gene transfer into almost all human cells and are thus not suitable for *in vivo* applications in gene therapy in which cell-specific gene delivery is required. We and others recently reported the generation of MLV-derived vectors pseudotyped by variants of the envelope glycoproteins (Env) of human immunodeficiency virus type 1 (HIV-1), thus displaying the CD4-dependent tropism of the parental lentivirus (Mammano *et al.*, 1997, *J. Virol.* 71, 3341–3345; Schnierle *et al.*, 1997, *Proc. Natl. Acad. Sci. USA* 76, 8640–8645). However, because of their HIV-1-derived envelopes these vectors are neutralized by HIV-specific antibodies present in some infected patients. To circumvent this problem, we pseudotyped MLV capsid particles with variants of Env proteins derived from the apathogenic simian immunodeficiency virus (SIVagm) of African green monkeys (AGM; *Chlorocebus pygerythrus*). Truncation of the C-terminal domain of the transmembrane protein was found to be necessary to allow formation of infectious pseudotype vectors. These [MLV(SIVagm)] vectors efficiently transduced various human CD4-expressing cell lines using the coreceptors CCR5 and Bonzo to enter target cells. Moreover, they were resistant to neutralization by antibodies directed against HIV-1. Therefore, [MLV(SIVagm)] vectors will be useful to study the mechanisms of SIVagm cell entry and for the selective gene transfer into CD4+ T-cells of AIDS patients.

INTRODUCTION

Selective gene transfer into CD4-expressing human T-cells is necessary for the treatment of a variety of immune dysfunctions, including severe combined immunodeficiency (SCID; Anderson, 1984; Blaese *et al.*, 1995) and AIDS (for review, see Yu *et al.*, 1994; Pomerantz and Trono, 1995) as well as T-cell lymphomas. Retroviral vectors derived from murine leukemia virus (MLV) are suitable for efficient gene transfer into human T-cells, because they mediate chromosomal integration of the delivered expression vector and have a low frequency of genetic recombination or mutation.

In many of the ongoing clinical trials employing retroviruses, the retroviral envelopes have been derived from amphotropic MLV. The surface glycoprotein gp70-SU of amphotropic MLV, which targets the cell surface receptor Ram-1 (Kavanaugh *et al.*, 1994; Kozak *et al.*, 1995; Eiden *et al.*, 1996), allows infection of almost all human primary cells and is therefore not suitable for gene transfer into a predetermined cell type. Consequently, gene transfer is generally performed *ex vivo* and involves purification of the target cells, gene transfer in tissue culture, and reinfusion of the genetically modified cells.

MLV-derived retroviral vectors selective for CD4-positive cells have been described previously. We and others (Mammano *et al.*, 1997; Schnierle *et al.*, 1997) pseudotyped MLV capsid particles with the envelope glycoproteins (Env) of human immunodeficiency virus type 1 [MLV(HIV-1)] (Indraccolo *et al.*, 1998) with those of the simian immunodeficiency virus from Rhesus macaques (*Macaca mulata*; [MLV(SIVmac)]). However, the use of [MLV(HIV-1)] in HIV-seropositive individuals is hampered by the presence of antibodies directed against HIV-1 envelope proteins, which are expected to significantly reduce transduction efficiencies. Although neutralization of [MLV(SIVmac)] vector particles by HIV-1-specific antibodies was not studied by Indraccolo *et al.* (1998), it is known from previous reports that sera from HIV-1-infected individuals cross-react with SIVmac (Blomberg *et al.*, 1990). In contrast, the simian immunodeficiency virus (SIVagm) from African green monkeys (*Chlorocebus pygerythrus*) is only poorly neutralized by HIV-1-specific antibodies (Cichutek and Norley, 1993). Furthermore, while SIVmac is pathogenic not only in its natural host but also in other primates (Simon *et al.*, 1994) and humans (Khabbaz *et al.*, 1994), SIVagm is apathogenic after natural and experimental infection of African green monkeys and other nonhuman primates (Beer *et al.*, 1998), making the Env proteins of this virus more favorable for the generation of gene-therapy vectors.

The [MLV(HIV-1)] vectors were constructed using the

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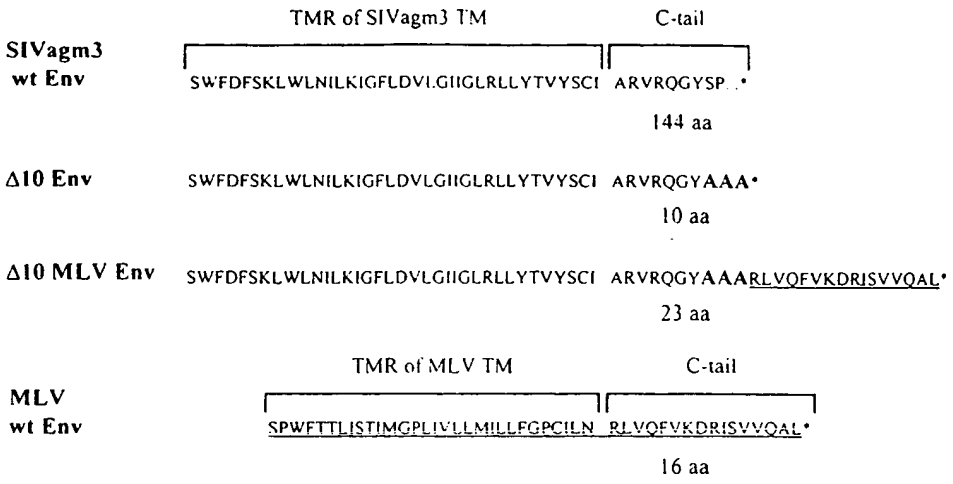

FIG. 1. C-terminal amino acid sequences of SIVagm3-TM, MLV TM, and recombinant TM-proteins. The amino acid sequences of the transmembrane region (TMR) and of the processed cytoplasmic domains (C-tails) are shown. Sequences derived from MLV are underlined. Larger letters printed in bold indicate amino acid residues encoded by the recombinant *Not*I-restriction sites. All other amino acids are encoded by SIVagm3.

surface envelope protein gp120-SU of a T-cell line adapted HIV-1 variant and a truncated transmembrane protein (TM; reviewed in Adachi *et al.*, 1986; Wilk *et al.*, 1992). In contrast, no formation of infectious pseudotype vectors was observed using the full-length TM-proteins of HIV-1. Curiously, no C-terminal truncation was reported to be necessary for SIVmac Env incorporation by MLV (Indraccolo *et al.*, 1998), pointing out considerable differences between the envelope glycoproteins of simian and human immunodeficiency viruses. In addition, Lindemann *et al.* (1997) have reported that fusion of the C-terminal amino acids of the MLV TM mediated enhanced incorporation of heterologous envelope glycoproteins derived from human spuma retrovirus (HSRV) into MLV-based capsid particles. We therefore wanted to assess whether these C-terminal regions of MLV TM would also support the incorporation of envelope glycoproteins derived from SIVagm into the respective vector particles.

The study revealed that truncation of the C-terminal domain of TM of SIVagm is required for the formation of infectious pseudotype vectors. The [MLV(SIVagm)] vectors specifically infected CD4+ human cells but, in contrast to [MLV(HIV-1)] vectors, were resistant to HIV-specific antibodies or any other inhibiting factor present in the sera of HIV-1-infected individuals.

RESULTS

Variant *env* genes of SIVagm are functionally expressed in TELCeB6/rev cells and mediate syncytia formation with CD4+ T-cells

The wild-type *env* gene of SIVagm encodes a 130-kDa surface glycoprotein (gp130-SU) and the transmembrane glycoprotein gp35-TM. Two *env* gene variants derived from wild-type SIVagm3mc *env* (Baier *et al.*, 1990) were constructed by C-terminal truncation of TM (see Fig. 1). These variants were designed to encode the full-length gp135-SU plus a truncated variant of TM, each comprising a C-tail of 10 (*Δ10 env*) and 23 (*Δ10MLV env*) amino acids, respectively, following the putative transmembrane region (TMR). As a result of the cloning strategy, a few additional amino acids unrelated to the wild-type TM were added to the C-termini (see Fig. 1, amino acids in bold). *Env* genes *Δ10 env* and *Δ10MLV env* encode 7 C-terminal amino acids derived from SIVagm and 3 residues generated by a recombinant *Not*I-restriction site within the respective *env* gene variant. In addition, *Δ10MLV env* includes parts of the MLV *env* gene to express a chimeric TM-protein terminated by amino acids derived from MLV TM, which presumably allow contact with the capsid proteins of the MLV core. The HIV-1 *rev*-positive, but *env*-negative, packaging cell line TELCeB6/rev constitutively producing capsid particles of MLV was used for the initial experiments to ensure sufficient expression of recombinant *env* gene constructs derived from SIVagm. This new packaging cell line allows efficient expression of *rev*-negative lentiviral *env* genes (data not shown).

TELCeB6/rev cells were transfected with plasmid DNA comprising the variant SIVagm *env* genes described above and cocultivated with Molt4.8 cells. After 3 days, very large syncytia were seen in cultures containing cells expressing *Δ10 env*, whereas all other cocultures, including those with wild-type SIVagm Env-positive cells, showed only small syncytia (data not shown). All syncytia observed were specifically stained in the SIVagm-specific immunoperoxidase assay, indicating that they were formed by expression of fusion-competent *env* gene products.

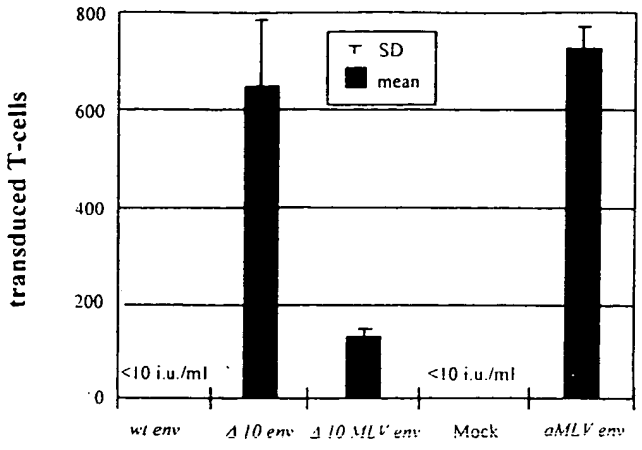

FIG. 2. Efficacy of pseudotype formation using variant SIVagm *env* genes. TELCeB6/rev cells were transiently transfected with the *env* genes indicated followed by cocultivation with Molt4.8 T-cells. Mock-transfected TELCeB6/rev cells served as negative controls; cells transfected with plasmid pHIT456 DNA encoding the *env* gene of amphotropic MLV (*aMLV env*) served as positive controls. T-cells were then stained using the X-gal assay. Transduction efficacies represent the results of three independent experiments.

SIVagm-derived *env* gene products encompassing C-terminally truncated TM allow pseudotype formation with MLV

To test [MLV(SIVagm)] particle formation and subsequent marker gene transfer, the cell line TELCeB6/rev was transfected with plasmid DNA encoding the respective SIVagm *env* gene variants or with plasmid pHIT456 encoding the amphotropic MLV *env* gene *(aMLV env)* as a positive control. Twenty-four hours later, the cells were cocultivated with 5×10^5 Molt4.8 T-cells in transwell culture plates, which prevent cell-to-cell contact but allow diffusion of vector particles to the target cells. After a further 2 days, the T-cells were expanded separately for an additional 2 days, and then X-gal- stained to detect marker-gene transfer. The transfected packaging cells were also immunostained to quantify the number of cells expressing lentiviral envelope glycoproteins to ensure comparable transfection efficiencies in all experiments.

No gene transfer was detected using the transfected *env* gene construct pRepwt env or the negative control cells (Fig. 2). In contrast, efficient gene transfer was detectable following transfection of *env* gene variants *Δ10 env* and *Δ10MLV env*, presumably as a result of infectious pseudotype particle formation. The number of transduced cells, and thus the titer of [MLV(SIVagm)] particles formed, using Δ10 Env (650 transduced cells) was similar to that seen using amphotropic MLV Env (720 transduced cells). Addition of the C-terminal amino acid sequences of MLV in construct pRepΔ10MLV env led to a decrease in infectious particle formation relative to that observed using the *env* gene variant *Δ10 env*.

[MLV(SIVagm)] vector particles specifically infect CD4+ cells, efficiently utilizing the coreceptors CCR5 and Bonzo

To establish a packaging cell line continuously producing [MLV(SIVagm)] vector particles, plasmid pRepΔ10 env, containing in addition to the SIVagm *env* gene variant a hygromycin resistance gene, was transfected into TELCeB6 cells. Following hygromycin B selection, more than 50 resistant cell colonies were randomly selected and shown by immunostaining to express the SIVagm *env* gene. These colonies were pooled and expanded further to give the packaging cell line Δ10Mix. Using anti-SIVagm serum and a protein G-gold-labeled conjugate, labeled vector particles derived from Δ10Mix cells were seen by electron microscopy (data not shown), indicating the presence of SIVagm-derived envelope glycoproteins. Neither the parental *env*-negative packaging cell line TELCeB6 nor TELCeB6 cells transiently transfected with pHIT456 DNA (encoding the amphotropic MLV *env* gene) produced particles stained with this procedure (data not shown).

These [MLV(SIVagm)] vector stocks harvested from Δ10Mix packaging cells were employed to transduce the human T-cell lines Molt4.8, C8166, and Jurkat as well as HeLa and HeLaCD4+ cells. All CD4-positive human cells were transduced by [MLV(SIVagm)] vector particles (Fig. 3). Titers in Molt4.8 and C8166 cells were 8 and 5×10^3 i.u./ml, respectively. Titers 2–4 were orders of magnitude lower in Jurkat and HeLaCD4+ cells, while no transduction of CD4-negative HeLa cells was detected.

To verify that transduction by [MLV(SIVagm)] vector

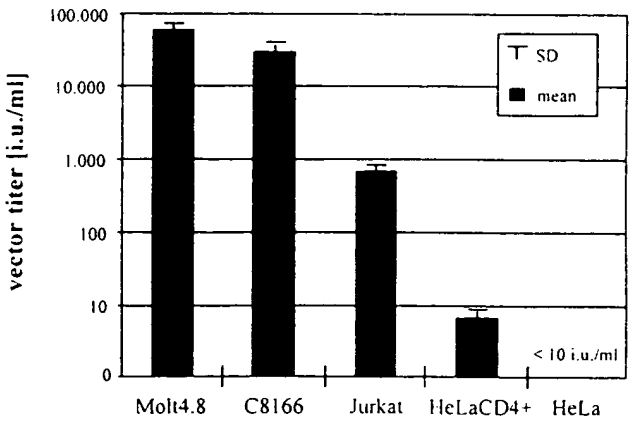

FIG. 3. Vector titers in various human CD4+ cell lines and HeLa cells. The cell lines indicated were transduced using different dilutions of a defined vector-containing supernatant derived from packaging cell line Δ10Mix. Infection of target cells was determined by X-gal staining 2 days postinfection. Titers shown represent the results of three independent experiments. Vector particles harvested from the *env*-negative packaging cell line TELCeB6 served as negative controls and were not able to mediate detectable gene transfer (< 10 i.u./ml).

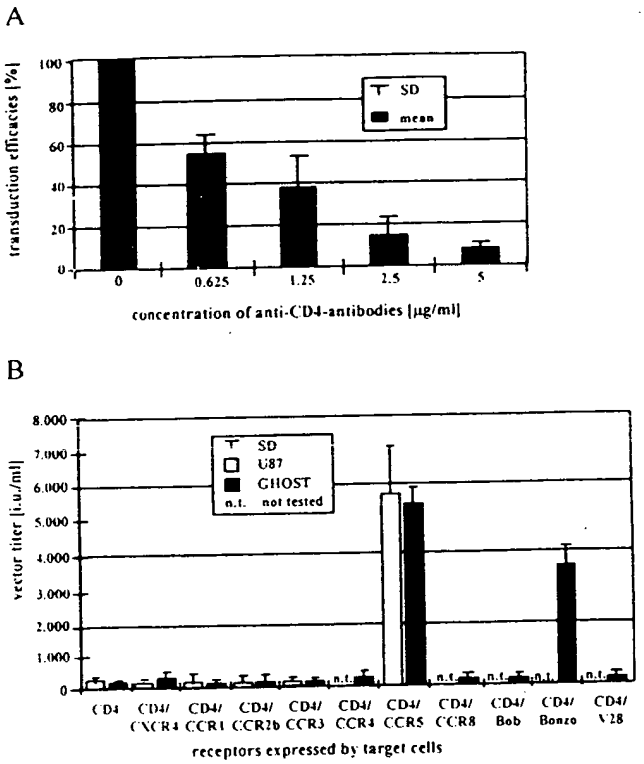

FIG. 4. CD4-specificity and coreceptor usage of [MLV(SIVagm)] vectors. (A) Molt4.8 T-cells were incubated in media containing different concentrations of the anti-CD4 antibody IOT4a and transduced with [MLV(SIVagm)] vectors. Transduction efficacies shown were determined using the X-gal assay and resulted from three independent experiments. (B) A panel of GHOST- and U87-derived cell lines expressing different coreceptors and CD4, or only CD4, was transduced by [MLV(SIVagm)] vectors and infection was revealed by X-gal staining 2 days postinfection. Titers shown represent the means of three independent experiments.

particles was CD4-dependent, transduction experiments were performed in Molt 4.8 cells in the presence of the monoclonal anti-CD4 antibody IOT4a, previously shown to inhibit HIV-1 infection (Sattentau *et al.*, 1986). Target cells were preincubated for 30 min with the antibody before [MLV(SIVagm)] vector particles were added. After 2 h, cells were washed and expanded for 3 days.Vector titers were then measured by X-gal staining. As shown in Fig. 4A, IOT4a dose-dependent inhibition of transduction was observed. In contrast, no influence on the gene transfer efficacy was detectable in the presence of 5 μg/ml of the control antibodies, directed against the nerve growth factor receptor and CD3, respectively (data not shown). Entry of the [MLV(SIVagm)] vector particles, therefore, depends on the presence and availability of the human CD4 receptor.

To elucidate the coreceptor dependence of the [MLV-(SIVagm)] vectors, two panels of cell lines (U87 and GHOST), each expressing human CD4 alone or CD4 plus one of several coreceptors described to allow cell entry of various HIV and SIV strains, were tested (Hill *et al.*, 1997; Cecilia *et al.*, 1998). In this investigation, 1×10 cells were transduced with various dilutions of [(MLV-(SIVagm)] vector-containing supernatant harvested from the packaging cell line Δ10Mix and vector titers were measured 2 days later. For both cell panels, the most efficient transduction was observed with cells expressing CD4 and the coreceptor CCR5 (Fig. 4B). GHOST cells expressing Bonzo were also transduced, however, significantly less efficiently. Besides CCR5, the coreceptors Bob and Bonzo were shown to be used by various other SIV strains (Deng *et al.*, 1997). It is noteworthy that all other U87 and GHOST cells, even those expressing only CD4, showed evidence of low-level transduction. For the GHOST-cell panel, a low level of Bonzo background expression was recently demonstrated by Edinger *et al.* (1998). As revealed by RT-PCR on RNA extracted from the U87 cell variants, we could detect Bob- and Bonzo-specific mRNA (data not shown). Thus, the low level of background transduction was probably due to Bonzo expression. Overall, CCR5 and Bonzo are the coreceptors used by the [MLV(SIVagm)] vectors. Similar coreceptor usage was observed during infection of U87 and GHOST cells with replication-competent SIVagm3mc (data not shown).

[MLV(SIVagm)] pseudotype vectors are resistant to neutralization by sera from HIV-1 infected individuals

SIVagm are only poorly neutralized by anti-HIV-1 antibodies (Cichutek and Norley, 1993) and to test whether [MLV(SIVagm)] vector particles are also unaffected, transduction experiments were performed in the presence of sera from asymptomatic HIV-1-infected donors previously shown to neutralize various T-cell line adapted and primary isolates of HIV-1: 5×10^3 i.u. of [MLV(SIVagm)] and of [MLV(HIV-1)] (Stitz *et al.*, 1998) were used to transduce Molt4.8 T-cells after incubation with the sera (diluted 1:20) for 1 h at 37°C. Two days later, as shown in Fig. 5, X-gal staining revealed that the titers of both pseudotyped vectors were slightly decreased by serum from a healthy donor (control serum). In contrast, anti-HIV-1 sera drastically reduced the titer of the [MLV(HIV-1)] vector particles, whereas no significant effect on the [MLV(SIVagm)] vector was seen.

DISCUSSION

Here we report that the gene products of SIVagm *env* gene variants encoding C-tails of 10 and 23 amino acids enabled formation of infectious [MLV(SIVagm)] vector particles. In contrast, the wild-type Env of SIVagm, comprising 144 C-terminal amino acids, was unable to mediate infectivity to MLV capsid particles. Thus, these results are in line with our previous findings on the generation of [MLV(HIV-1)] pseudotype vectors (Mammano *et al.*, 1997; Schnierle *et al.*, 1997) but are, however, in contrast to the studies of Indraccolo *et al.* (1998).

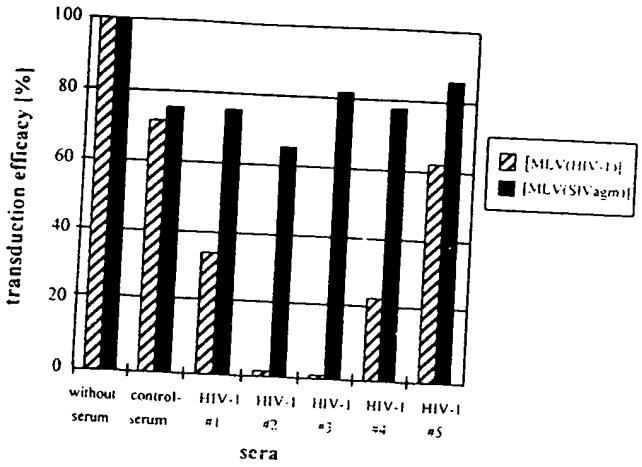

FIG. 5. Neutralization of pseudotype vectors by sera from healthy and HIV-1-infected donors. Pseudotype vectors were incubated with serum from a healthy (control serum) or five HIV-1-infected donors (HIV 1 to 5) and then used to transduce Molt4.8 T-cells. Infection was determined employing the X-gal assay. Transduction efficacies represent the means of two independent experiments.

Curiously, Indraccolo and his coworkers (1998) described comparable vector titers using the unmodified wild-type and a C-terminally truncated variant of SIVmac Env protein, respectively. Possibly, the three-dimensional structure of the C-terminal domain of the SIVmac TM protein differs from that of the SIVagm and HIV-1 TM proteins and is therefore more easily incorporated into the MLV capsid particles. An unexpected finding of our experiments was that adding the C-terminal amino acids derived from TM of amphotropic MLV to the truncated SIVagm TM C-tail did not increase vector titers but even reduced them about fivefold. From studies on HIV-1 assembly, it is known that contact between the C-tail of Env and the capsid facilitates incorporation of Env proteins (Andreassen *et al.*, 1992; Yu *et al.*, 1992; Bugelski *et al.*, 1995; Egan *et al.*, 1996; Lee *et al.*, 1997) and Lindemann *et al.* (1997) demonstrated that the fusion of MLV-derived C-tails to the Env proteins of human spuma retrovirus (HSRV) significantly enhanced the efficiency of infectious pseudotype vector formation. It is a matter of speculation that these contradictory findings might result from differences in the tertiary structure of HSRV-TM and SIVagm3-TM. Further constructs need to be tested to resolve this difference.

Alternatively, the enhanced ability of variant Δ10 Env to induce large syncytia as compared to Δ10MLV Env, might explain its higher infectivity. The increase of the fusion ability of lentiviral Env proteins by truncation of the TM C-terminus was demonstrated before. HIV-1 Env gene variant Tr712 was used for the production of infectious [MLV(HIV-1)] vector particles (Wilk *et al.*, 1992; Schnierle *et al.*, 1997). Similarly, Zingler and Littman (1993) observed enhanced infectivity of SIVmac239 pseudotyped with C-terminally truncated Env-variants compared to wild-type envelope glycoproteins. In addition, the increase of the fusion capability of MLV Env by truncating the C-tails during virion maturation is well characterized (Rein *et al.*, 1994).

Using the Env variant Δ10 Env, we established a stable pseudotype packaging cell line termed Δ10Mix to generate [(MLV(SIVagm)] pseudotype vector stocks. The [MLV(SIVagm)]-pseudotyped vectors allowed efficient transduction of human T-cells with titers of up to 8×10^5 i.u./ml. In contrast, [MLV(SIVmac)] pseudotype vector particles (Indraccolo *et al.*, 1998) were described to be less efficient, yielding maximum titers of 3×10^4 i.u./ml. More important, and in contrast to [MLV(HIV-1)] vector particles, the [MLV(SIVagm)] vectors were demonstrated to be resistant to neutralization by sera from HIV-1-infected individuals, most likely due to the resistance against neutralizing HIV-1-specific antibodies.

Transduction mediated by the [MLV(SIVagm)] pseudotype vectors was shown to be CD4-dependent by inhibition of vector-mediated gene transfer in the presence of CD4-specific antibodies. Like SIVagm3mc itself (data not shown), [MLV(SIVagm)] vector particles were demonstrated to efficiently use the coreceptors CCR5 and Bonzo. CD4 and CCR5 are expressed on human primary T-cells and macrophages (Alkhabit *et al.*, 1996; Deng *et al.*, 1996; Raport *et al.*, 1996; Samson *et al.*, 1996), and are used by HIV-1 for host cell entry (Alkhabit *et al.*, 1996; Choe *et al.*, 1996; Dragic *et al.*, 1996; Raport *et al.*, 1996). Besides macrophages, CD4+/CCR5+ T-cells are believed to function as the natural host cell reservoir for M-tropic HIV-1 strains that are responsible for the initial infection in humans. Therefore, [MLV(SIVagm)] vector particles may be useful for transduction of human CD4+ CCR5+ T-cells with therapeutic genes encoding HIV-specific ribozymes, intrabodies, or antisense RNA (Yu *et al.*, 1994). Efforts will be made to test the new vectors for their ability to transduce primary human and primate CD4+ cells and to establish more efficient packaging cells as a prelude to further studies *in vivo*. In addition, SIVagm Env variants are currently developed to allow the generation of [MLV(SIVagm)] vectors specific for CD4+ CXCR4+ cells. The resistance of such vectors to neutralization by sera from HIV-1-infected donors may make them ideal vehicles for specific therapeutic gene transfer in HIV-1-infected patients, offering new possibilities for *in vivo* gene therapy against HIV infection and AIDS.

MATERIALS AND METHODS

Plasmid constructs

Plasmids were purified from transformed *Escherichia coli* strain Top 10F'. The *env* gene variants of SIVagm3mc were generated using standard PCR and fusion-PCR techniques (Sambrook, 1989) and were inserted into the multiple cloning site of plasmid pRep 4 (Invitrogen, Leek,

Netherlands) via *NheI*/*XhoI*. Env gene expression is facilitated by the 3'LTR of RSV (Rous sarcoma virus) and a polyadenylation signal derived from simian virus 40 (SV40). (Primer-sequences are available on request.) The plasmid pRepwt env encompasses the entire complement of *rev* and *env* genes of SIVagm3mc (Baier *et al.*, 1990; Dittmar *et al.*, 1995). The plasmid pRepΔ10 env is identical to pRepwt env, except that the intracellular domain (C-tail) of the transmembrane protein (TM) is truncated to a total extent of 10 amino acid residues. This was achieved by recombinant PCR, inserting a recombinant *NotI*-restriction site followed by a stop codon. The plasmid pRepΔ10MLV env was generated by inserting sequences encoding parts of the C-tail of MLV into the recombinant *NotI*-restriction site. The amino acid sequences of the C-tails encoded by the plasmids mentioned earlier are shown in Fig. 1. The plasmid pHIT456 encoding the *env* gene of amphotropic MLV (*aMLV env*, kindly provided by A. J. Kingsman; University of Oxford, Cambridge, U.K.) has been described elsewhere (Soneoka *et al.*, 1995).

Cell lines

The T-cell lines Molt4.8, C8166 and Jurkat were grown in RPMI 1640 medium containing 10% fetal calf serum (FCS). The adherent cell lines HeLa, HeLaCD4+ (ADP047; reviewed in Chesebro *et al.*, 1991), and the derivatives of U87 and GHOST (both cell line panels provided by D. Littmann, New York University Medical Center, New York; reviewed in Hill *et al.*, 1997; Cecilia *et al.*, 1998), as well as the packaging cell lines TELCeB6 (kindly given by Y. Takeuchi and F.-L. Cosset, Chester Beatty Laboratories, London, U.K., reviewed in Cosset *et al.*, 1995) and TELCeB6/rev, were grown in Dulbecco's Modified Eagles Medium (DMEM; GIBCO/BRL, Eggenstein, Germany) supplemented with 10% FCS (GIBCO/BRL).

The *env*-negative packaging cell line TELCeB6 (Cosset *et al.*, 1995), providing the *gag/pol* gene products of MLV in *trans* and expressing a ψ-positive, β-Gal-encoding expression vector, was employed to generate pseudotype vector particles, because this cell line constitutively releases a large amount of MLV core particles. The cell line TELCeB6/rev is a cell clone derived from TELCeB6 cells, which additionally expresses the *rev*-gene of HIV-1 (construct pCMV-rev/hyg; reviewed in Stitz *et al.*, 1998). Transfections of the packaging cell lines TELCeB6/rev and TELCeB6 were performed employing Lipofectamine (GIBCO/BRL), according to the manufacturer's instructions. Selection of hygromycin-resistant colonies after transfection of the plasmids derived from pRep 4 was carried out in medium containing 200 μg/ml hygromycin B (Sigma, Deisenhofen, Germany).

Expression and membrane fusion capacity of the SIVagm envelope glycoproteins

TELCeB6/rev cells were transfected with the plasmids encoding the recombinant *env* genes of SIVagm3mc. On the following day, the transfected packaging cells were overlaid with Molt4.8 T-cells and cocultivated for 2 days. Cultures were then incubated with ice-cold methanol for 15 min. After repeated washing with PBS, blocking buffer (PBS/1% BSA) was added for 1 h. Cells were then washed again and incubated with an anti-SIVagm serum diluted 1:400 (Nem 170). After further washing, cells were incubated with peroxidase-conjugated protein-G (Biorad, Krefeld, Germany). Finally, antigen-containing cells were visualized by addition of substrate buffer as described previously (Schnierle *et al.*, 1997).

Cocultivation experiments

TELCeB6/rev cells were seeded in double-chamber six-well tissue culture dishes (Transwell, Costar, Cambridge, MA) and transfected with the respective plasmids. Two days posttransfection, Molt4.8 cells were added to the upper chamber, allowing the free diffusion of vector particles but preventing cell-to-cell contact between the packaging cells and target T-cells. After 2 days, Molt4.8 cells were separated from the transfected packaging cells and expanded for a further 2 days. Transduced T-cells were then stained using the X-gal assay as described elsewhere (Sanes *et al.*, 1986).

Viral infection, determination of titers, and neutralization experiments

Adherent target cells were seeded in six-well culture dishes at a density of 2×10^5 cells/well and T-cells at 1×10^5 cells/well. Serial dilutions of cell-free (0.45 μm, filtered) vector containing supernatants were added, incubated for 2 h, and washed off. After 2 days, the cells were tested for β-Gal activity, stained cells were counted, and viral titers expressed as infectious units per milliliter (i.u./ml). To demonstrate that vector entry into the target cells was mediated by specific binding of the pseudotype vector particles to the cellular receptor CD4, transduction of permissive T-cells was performed in the presence of the neutralizing (Sattentau *et al.*, 1986) CD4-specific monoclonal antibody IOT4a (Dianova, Hamburg, Germany), antibodies directed against the human nerve growth factor receptor (αNGFR; Boehringer, Mannheim, Germany), and CD3 (OKT-3; OrthoDiagnostics, Germany), respectively. Susceptibility to neutralization by sera from HIV-1-infected donors was tested byincubating 5×10^3 i.u. vector particles with or without sera (diluted 1:20) from HIV-1-infected or uninfected donors for 1 h at 37°C in a total volume of 1 ml. Samples were then used to transduce Molt4.8 T-cells as described. Vector titers were estimated after X-gal staining of transduced cells.

Electron microscopy

Confluent cultures of the packaging cell lines were treated with fixation buffer (PBS/2% formaldehyde) for 1 h at 4°C, repeatedly washed with PBS, and finally incubated with 1:100 dilutions of anti-SIVagm serum (Nem 170) for 1 h at 37°C. After further washing, a 1:50 dilution of gold particle-conjugated protein-G (Bio Cell, Cardiff, U.K.) was added and left for 1 h at 37°C. After extensive washing with PBS, cells were embedded in epoxyd according to standard procedures (Luft, 1964).

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High-titer retroviral pseudotype vectors for specific targeting of human CD4-positive cells

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We (Schnierle & Stitz *et al.*, 1997) previously reported the generation of packaging cell lines that produced MLV(HIV-1) pseudotype vector particles at moderate titers of up to 9 x 10^4 infectious units per millilitre (i.u./ml). New packaging cell lines have now been established that enable us to create MLV(HIV-1) pseudotype vector preparations reaching a titer of more than 10^8 i.u./ml. Using these vectors, stimulated human primary CD4-positive T-cells were efficiently transduced.

Key words: gene therapy; HIV-1; MLV-vectors; cell targeting; CD4-expressing human cells; pseudotyping; vector concentration procedures

INTRODUCTION

An ideal gene therapy protocol would comprise direct application of gene delivery vehicles (e.g. viral vector particles) into suitable sites of the patient's body, e.g. into the blood stream. Such gene delivery systems probably would have to allow 1) cell specific expression of the foreign gene in the cell type to be genetically modified, 2) long-term expression of

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the delivered gene, and 3) safe and easy clinical handling which should also be cost-effective.

The retroviral vectors which are until today most frequently employed in clinical trials are derived from the well characterised amphotropic murine leukaemia virus (MLV). These vector particles are able to transduce proliferating cells of rodent and human origin expressing the cellular receptor Ram-1. Besides serving as the receptor for amphotropic MLV, Ram-1 functions as a phosphate-channel (Kavanaugh *et al.*, 1994; Kozak *et al.*, 1995; Eiden *et al.*, 1996). As Ram-1 is expressed in all human cell species, vectors derived from amphotropic MLV are not suitable for *in vivo* gene delivery because unspecific transduction of cells not intended to be genetically modified is likely to occur. The unspecific transduction of cells from irrelevant tissues will presumably require higher vector doses to achieve transduction efficiencies in the target cell population that would allow the reconstitution of tissue specific functions previously disrupted by genetic defects. Higher vector doses will most likely result in a stronger immune response against the viral vector particles and thus, will make repeatable efficacious vector application less likely, as vector particles will probably be increasingly neutralised by antibodies directed against the vector proteins. Moreover, the risk of malignant cell transformation as a consequence of the unspecific integration of vector sequences into the host cell genome (insertional mutagenesis) may increase, if irrelevant tissues are transduced. To avoid these major drawbacks, efforts have been undertaken during the past years to develop MLV-derived vectors with a narrowed host cell range.

To alter vector tropism, several strategies have been invented, including the use of chimeric envelope proteins that comprise antibody fragments or receptor ligands. These modifications are aimed at allowing the respective vector particles to specifically bind to the antigen or the receptor corresponding to the antibody or the ligand that has been fused to Env proteins, and then to specifically enter a certain cell type displaying the

cognate antigen or receptor. These approaches have been shown to be successful, but the gene transfer efficiencies reached so far have been relatively low (Chu *et al.*, 1994; Somia *et al.*, 1995; Ager *et al.*, 1996).

The cell tropism of enveloped viruses or viral vector particles is determined at the level of entry by the envelope proteins, which allow binding to as well as uptake into the cell. The tropism of a given virus can be conferred to a viral vector particle by incorporating heterologous envelope proteins into the virion, a procedure termed pseudotyping. This may result in the generation of virions with an altered host cell range. A number of MLV-derived pseudotype vector particles have been developed in recent years, among them several that were intended to further optimize gene delivery for the purpose of gene therapy. For example, capsid particles derived from MLV which are pseudotyped using the envelope proteins of the gibbon ape leukaemia virus (GaLV) or the G-protein of the vesicular stomatitis virus (VSV) are often used in today's *ex vivo* transduction of haematopoetic stem cells (Kalle *et al.*, 1994). These MLV(VSV-G) and MLV(GaLV) pseudotype vector particles seem to be superior with regard to the achievable gene delivery efficiency compared to amphotropic MLV-vectors, probably because the cellular receptors used by GaLV and VSV to enter the host cell are most likely expressed at higher levels than the amphotropic receptor Ram-1 (Richardson *et al.*, 1996). Pseudotype vector particles that display the envelope proteins of the feline endogenous virus RD114 (Cosset *et al.*, 1995) are resistent against inactivation by human complement and are therefore believed to be more stable under *in vivo* conditions. Remarkably, none of the vector particles mentioned above show a narrowed host range to a limited cell species of human origin and are therefore only suitable for *ex vivo* gene delivery. Further pseudotype vectors derived from MLV have been developed, but are not necessarily suitable for gene therapy rather than for studying virological phenomena such as receptor choice or cell entry mechanisms of

the parental viruses the envelope proteins were derived from (Vile *et al.*, 1990; Landau *et al.*, 1992).

We and others (Schnierle & Stitz *et al.*, 1997; Mammano *et al.*, 1997) decided to exploit the restricted tropism of lentiviruses by incorporating lentiviral envelope glycoproteins into MLV capsids. This was shown to result in the generation of retroviral pseudotype vector particles that selectively transduce CD4-expressing human cells and accordingly promise to be valuable tools for gene therapy. Diseases that affect CD4+ cells could be possible targets for gene transfer using MLV(HIV-1) vector particles. These include severe combined immunodeficiency (SCID) (Anderson *et al.*, 1984; Blaese *et al.*, 1995) and AIDS (reviewed in: Yu *et al.*, 1994; Pomerantz *et al.*, 1995).

MATERIALS AND METHODS

Bacteria and plasmids

All plasmids were purified from the *E. coli*-strain DH10B using plasmid purification kits (Diagen, Hilden, Germany). The plasmid pTr712 encoding a truncated Env-variant of HIV-1 was described elsewhere (Wilk *et al.*, 1992). The plasmid pCMV-*rev*/hyg was derived from pCMV-*rev* (Lewis *et al.*, 1990) by insertion of a DNA fragment encompassing the hygromycin B gene via *Sph I*, which was amplified by PCR from DNA of the plasmid pRep4 (Invitrogen, Leek, Netherlands) by a standard protocol using the following oligonucleotides: Hygro 5'-Sph I (+), i.e. 5'-CATGCATGCCTGCTTCATCCCCGTGGCCCG-3' and Hygro 3'-Sph I (-), i.e. 5'-ACATGCATGCCCAGACCCCAGGCAACG CCC-3'.

Cells and transfections

The *env*-negative MLV-derived packaging cell line TELCeB6 (Cosset *et al.*, 1995) was kindly provided by F.-L. Cosset (Centre National de la Recherche Scientifique, Lyon, France) and Y. Takeuchi (Institute of Cancer Research, London, UK). TELCeB6 cells express the *gag/pol*-genes

of MLV and the transfer vector MFGlnslacZ (Ferry *et al.*, 1991) containing the reporter gene *lacZ* that encodes the beta-galactosidase of *E. coli*. CD4+ HeLa cells (ADP047; Chesebro *et al.*, 1991) and the T-cell lines Molt4.8 (175), Jurkat (177) and C8166 (404) were purchased from the NIH-AIDS Research and Reference Reagent-Program. All adherent cell lines were kept in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal calf serum (FCS, Biochrom KG, Berlin, Germany). The T-cell lines were expanded in RPMI 1648 containing 10% FCS. For the transfection of TELCeB6 and K52S cells, we employed the reagent Lipofectamine (Gibco/BRL, Eggenstein, Germany) following the manufacturer's instructions. Selection of transfected cells was performed using 800µg/ml G418 and 200µg/ml hygromycin B (Boehringer, Mannheim, Germany) respectively. Primary human peripheral blood mononuclear cells (PBMCs) were prepared from blood samples of healthy donors by Ficoll-gradient-centrifugation (Ficoll-Histopaque, Sigma, Deisenhofen, Germany). Cells were stimulated using 20 u/ml IL-2 (Eurocetus, Ratingen, Germany) and 1 µg/ml PHA (Murex Biotech, Dartford, UK). Monoclonal αSDF-1β-antibodies were purchased from R&D-System (Wiesbaden, Germany).

Determination of vector titers

The calculation of vector titers and the X-Gal staining procedure were described previously (Schnierle & Stitz *et al.*, 1997). Endpoint titrations of pseudotype vector stocks were performed using various dilutions of a total volume of 1 ml to infect 1 x 10^6 suspension cells that were washed and pelleted prior to transduction. Adherent target cells were seeded at a density of 2 x 10^5 cells per six-well (Nunc, Wiesbaden, Germany) 24 hours prior transduction. All target cells were exposed to vector particles for 2 hours followed by washing with PBS. Transduced cells were then further expanded for two days before *lacZ*-positive cells were detected by X-Gal staining.

Electron microscopy

Confluent cultures of the packaging cell line K52S were treated with fixation buffer (PBS / 2 % formaldehyde) for one hour at 4°C and then repeatedly washed with PBS and incubated with 1:500 dilutions of an anti-HIV-1 serum from a HIV-1-infected donor for one hour at 37°C. After further washing, a 1:50 dilution of gold particle-conjugated anti-human IgG (Biocell, Cardiff, UK) was added and left for one hour at 37°C on the samples. After extensive washing with PBS, cells were embedded into epoxyd according to standard procedures (Luft *et al.*, 1964).

Generation of vector stocks and ultrafiltration

Vector-containing supernatants (15-20 ml per 250 cm2 flask) were generated by incubation of confluent cultures of packaging cells for 6 to 12 hours with respective cell culture media. Supernatants were pooled and contaminating cells were removed by filtration (0.45 µm filter). These samples were then stored or further concentrated by ultrafiltration. We used Centriprep-devices (Amicon, Beverly, 01915 MA, USA) with membranes that are pervious to molecules of a weight smaller than 30 kDa or 50 kDa according to the manufacturer's instructions. Usually, 17 ml vector-containing supernatants were reduced to a final volume of 1 ml by this procedure. Untreated and concentrated vector stocks were stored in liquid nitrogen.

RESULTS

Establishing high-titer packaging cell lines

The previously established MLV(HIV-1) vector producer cell lines TELCeB6/pTr712-K14 and TELCeB6/pTr712-9 (Schnierle & Stitz *et al.*, 1997) derived from the *env*-negative packaging cell line TELCeB6 (Cosset *et al.*, 1995) and transfected with HIV-1 *env*-gene variant pTr712 (Wilk *et al.*, 1992) enabled us to prepare vector stocks reaching titers of up to 10^5

i.u./ml. To test whether more efficient packaging cell lines could be established, we transfected plasmid pTr712 into TELCeB6 cells followed by G418-selection two days later. Further 10 days later, 200 cell clones were picked and screened for infectious vector particle release by titration of pseudotype vector containing supernatants in CD4+ HeLa cells (Chesebro *et al.*, 1991) that stably express human CD4. Cell clone K52S produced the highest vector titers and was expanded. Endpoint titrations repeatedly performed in CD4+ HeLa cells and various T-cell lines (Molt4.8, Jurkat and C8166) revealed pseudotype vector titers of up to 1.6 x 10^6 i.u./ml (Table 1).

Table 1. MLV(HIV-1) vector titers of supernatants from the packaging cells K52S and K52S/R20 in CD4-positive cell lines and HeLa cells*

Target cells	Packaging cells	
	K52S	K52S/R20
CD4+ HeLa	1.6 x 10^6 **	5 x 10^6 **
HeLa	n.d.	n.d.
Molt4.8	9 x 10^5	n.t
C8166	9 x 10^5	nt
Jurkat	5 x 10^5	n.t

* Gene transfer efficiencies were determined by X-Gal staining. Transduction of standard HeLa cells was not detected (n.d.). Titers shown resulted from three independent experiments (n.t. = not tested).
** i.u./ml

All following experiments described here were performed employing vector stocks prepared from the new packaging cell line K52S. The packaging cell line K52S showed no significant loss in its capacity to produce high-titer vector stocks over a period of several months. By immunostaining using HIV-1-specific anti-sera from HIV-1-infected human donors as previously described (Schnierle & Stitz *et al.*, 1997) only about 40% of the K52S cells were found to express the variant envelope

proteins of HIV-1. Assuming that the titer of infectious vector particles should correlate with the percentage of *env*-positive packaging cells, we attempted to increase the titer by subcloning the K52S cells. To avoid the time-consuming procedure of biological subcloning and in attempt to enhance the expression levels of the HIV-1-derived envelope glycoproteins, plasmid pCMV*rev*/hyg encoding the HIV-1 *rev*-gene was transfected into K52S cells followed by hygromycin B-selection. Fifty resistant cell clones were screened for efficient vector particle release as described above. The most productive clone termed K52S/R20 was shown to produce three-times higher infectious vector titers. More than 95% of the K52S/R20 cells were found to express the HIV-1-derived envelope glycoproteins as shown by Env-specific immunostaining (data not shown).

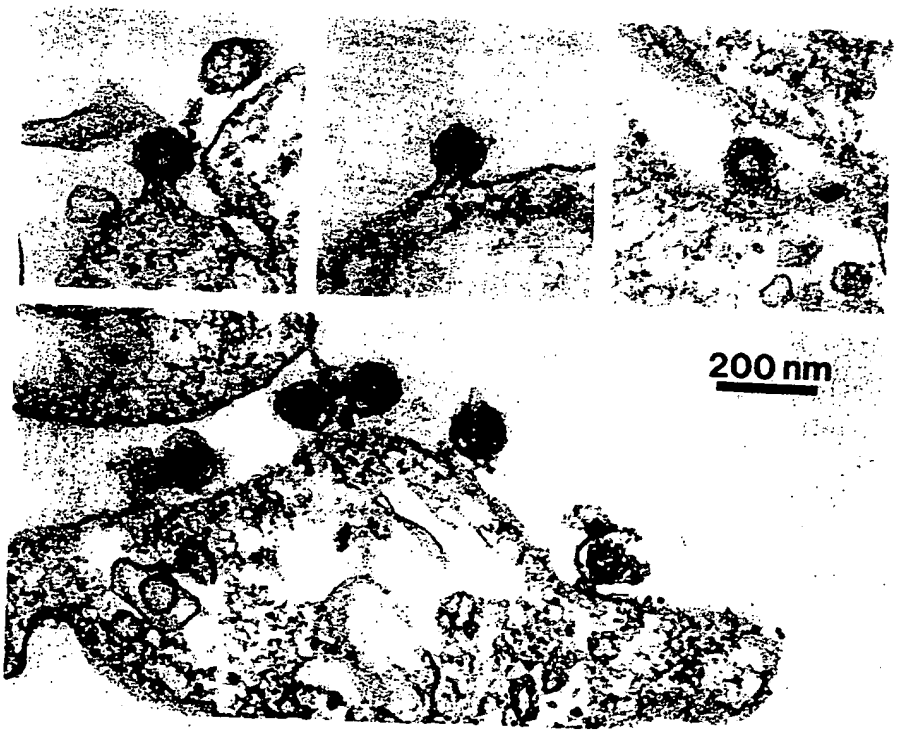

Figure 1. Electron microscopic images of MLV(HIV-1) vector particles. K52S packaging cells were fixed and stained employing an αHIV-1 serum and a human IgG-gold conjugate. Specifically labeled vector particles were found, demonstrating the presence of HIV-1-derived envelope proteins in the MLV-derived virions.

Morphology of MLV(HIV-1) pseudotype vector particles

To directly demonstrate the presence of HIV-1-derived envelope proteins in MLV-capsid particles, we prepared confluent cultures of K52S cells for electron microscopy. Specific labelling of the envelope proteins was performed using polyclonal antibodies from a HIV-1-infected human donor and α-human-IgG-gold conjugates as described in materials and methods. The electron microscopic images revealed C-type retroviral particles specifically labelled by anti-HIV-1-antibodies (Fig. 1). This showed the presence of HIV-1-derived envelope glycoproteins in the vector particles released from packaging cell line K52S.

MLV(HIV-1) pseudotype vectors produced by the K52S packaging cell line use the CXCR4 co-receptor during entry into CD4-positive cells

Especially for *in vivo* use, it is indispensable to exactly characterize the tropism of the vector particles to be used. As plasmid pTr712 employed to establish packaging cell line K52S encoding, in addition to the truncated transmembrane protein (TM), the gp120-SU of HIV-1 isolate BH10 known to be T-cell tropic (Wong-Staal *et al.*, 1985; Gene Bank accession numbers M15654, K02008, K02009, K02010), the respective MLV(HIV-1) pseudotype vector particles were thus expected to mediate CXCR4-dependent transduction of CD4-positive cells. To experimentally verify this hypothesis, we used a panel of cell lines (kindly provided by D.R. Littman, Skirball Institute, New York, through E.M. Fenyö, Karolinska Institute, Stockholm, Sweden) derived by Hill *et al.* (1997) from the megaglioma-asterocyte cell line U87 as target cells for transduction. In contrast to the parental U87 cells, the panel of U87.CD4 cell lines expresses either human CD4 alone or in conjunction with one of the chemokine receptors known to function as co-receptors for HIV-1 infection (reviewed by D'Souza *et al.*, 1996). 1 x 10^5 cells were used as target cells and transduced using MLV(HIV-1) vector containing supernatants of K52S cells. The X-Gal

assay performed two days later revealed the exclusive transduction of U.87 cells expressing CD4 and the chemokine receptor CXCR4 (Fig. 2).

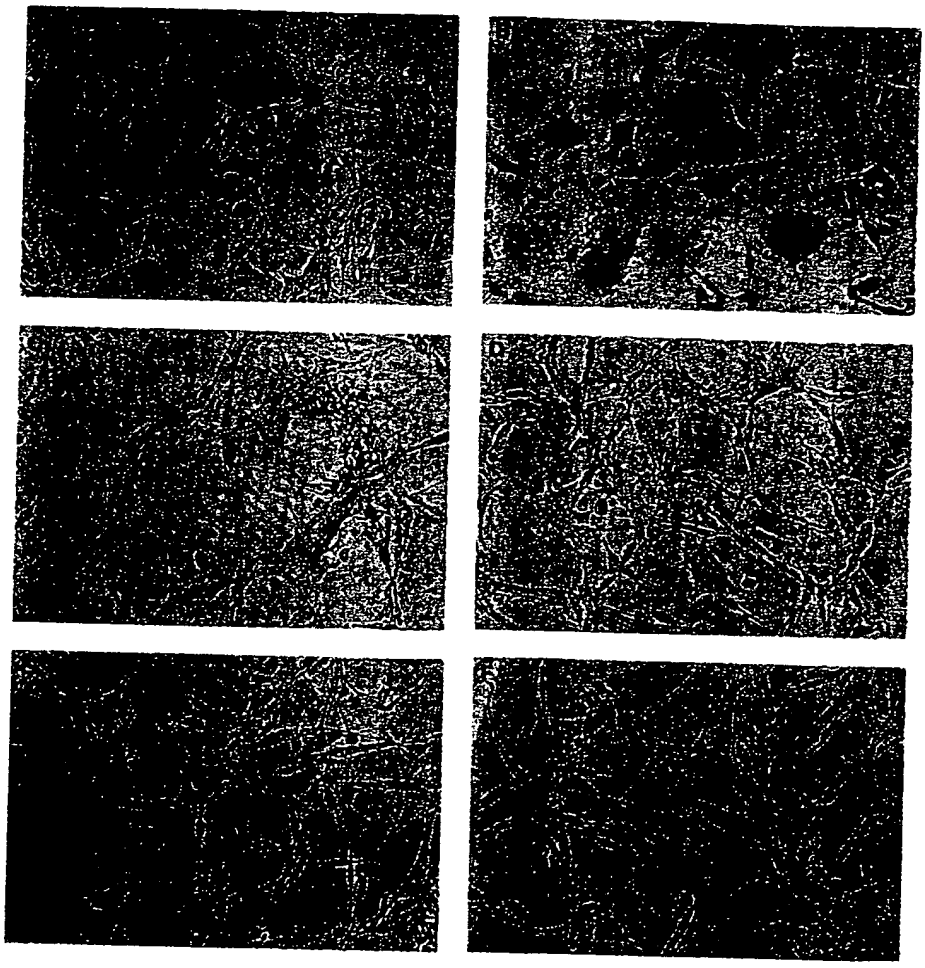

Figure 2. Co-receptor usage of MLV(HIV-1) vector particles. A panel of U87.CD4-derived cell lines was transduced by MLV(HIV-1) vector particles prepared from cell culture supernatants of K52S packaging cells. Successful gene delivery was revealed by X-Gal staining. Obviously, the respective vector particles use the co-receptor CXCR4 (B) to enter the target cells. No transduction of cells expressing any of the other co-receptors was detected.

A) U87.CD4 / -; B) U87.CD4 / CXCR4;
C) U87.CD4 / CCR1; D) U87.CD4 / CCR2b;
E) U87.CD4 / CCR3; F) U87.CD4 / CCR5.

No transduction of the U87.CD4 cells expressing any other of the co-receptors was detected. It was thus demonstrated that the MLV(HIV-1) vector particles retained the tropism of the parental T-cell tropic HIV-1-isolate BH10, from which the surface protein gp120-SU was derived from.

Optimising MLV(HIV-1) vector particle concentration

It is known that pseudotype vector particles containing the G-protein of VSV are very stable and can be efficiently concentrated by ultrafiltration or by ultracentrifugation. It was therefore also attempted to further increase the infectious titer of vector stocks by applying simple concentration procedures assuming sufficient stability. Seventeen ml of MLV(HIV-1) vector-containing supernatants obtained from confluent cultures of K52S packaging cells were usually concentrated to 1 ml using a single centrifugation step as described in materials and methods. The concentrated and, as controls, the untreated vector preparations were subsequently used to transduce C8166 T-cells. Transduction was detected by X-Gal staining two days post infection. In addition, differently conditioned cell culture media were compared with regard to their influence on vector production by the packaging cells. During production of vector containing supernatants, the packaging cells were cultured in DMEM with 10% FCS, glutamine and NSP (VZ), DMEM with glutamine and NSP (VZ-FCS) and DMEM without any supplements (pure), respectively. Unconcentrated and vector preparations ultrafiltrated using Centriprep30™- and Centriprep50™-devices were used in parallel to transduce C8166 T-cells. As shown in Fig. 3, the highest vector titers were reached using DMEM without any supplements during transduction of the unconcentrated vector particles. In contrast, the ultrafiltrated and thus concentrated packaging cell supernatants retained the highest vector titers when generated with complete cell culture media (VZ). This suggested that VZ allows the packaging cells to produce the largest amount of vector particles, but may somehow inhibit vector infectivity. The best vector preparations obtained using the described

concentration procedures yielded vector stocks with titers of up to 2 x 10^8 i.u./ml (data not shown).

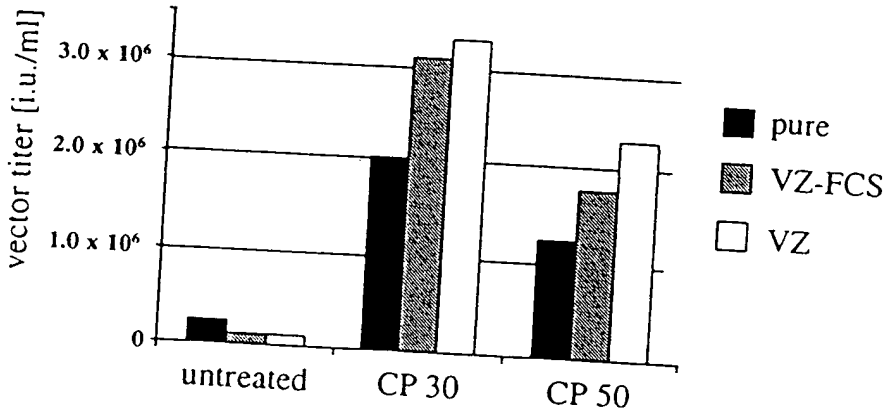

Figure 3. Concentrating vector particles by ultrafiltration. Supernatants of the packaging cell line K52S were prepared using different culture media: pure DMEM without any supplements (pure), DMEM supplemented with glutamine and NSP (VZ-FCS) and DMEM supplemented with glutamine, NSP and 10% FCS (VZ). Data shown resulted from one representative experiment.

Transduction of primary T-cells

To test the potential of MLV(HIV-1) vector particles to mediate gene delivery into primary T-cells, experiments were performed using human peripheral blood mononuclear cells (PBMCs). PBMCs were obtained from healthy human donors by Ficoll gradient centrifugation. The cells were stimulated by IL-2 and PHA for 48 hours. Three separate transduction protocols were designed. A portion of the stimulated cells was cultured in the presence of 10 µg/ml monoclonal antibody directed against the CXCR4-ligand human stromal derived factor 1β (SDF-1β). The antibodies were added 4 or 24 hours prior to transduction. In each case, αSDF-1β-antibodies were also present during transduction (5 µg/ml). In parallel, stimulated PBMCs were transduced in the absence of additional αSDF-1β.

Transductions were performed employing 1 x 10^6 PBMCs and 1 x 10^5 i.u. of MLV(HIV-1) vector particles titrated on CD4+ HeLa cells. The transduced target cells were expanded for 2 days before staining of lacZ-positive cells. Pre-incubation (24 h) of stimulated PBMCs with αSDF-1β -antibodies led to a 15-fold increase of transduction efficiency compared to stimulated PBMCs without αSDF-1β-pre-incubation (Fig. 4).

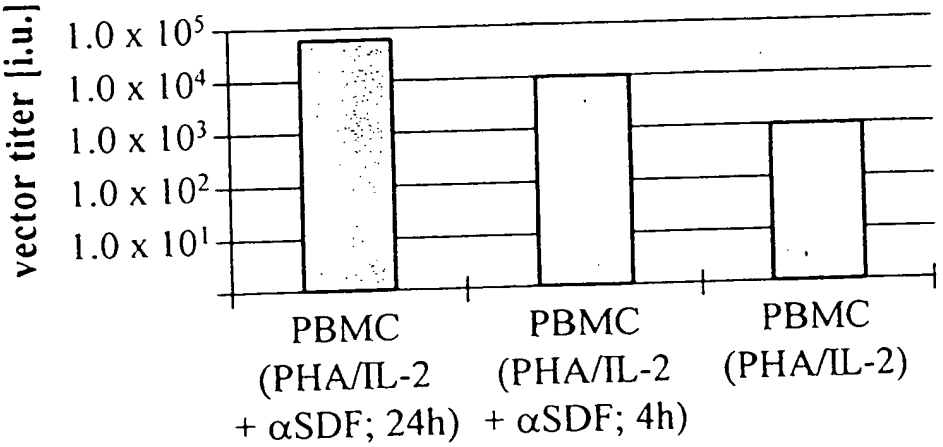

Figure 4. Transduction of peripheral blood mononuclear cells (PBMCs). Cells were stimulated by PHA and IL-2 for two days. A part of these cells was directly transduced by the respective MLV(HIV-1) vector particles, whereas some cells were pre-incubated in the presence of αSDF-1β-antibodies 4 or 24 hours prior transduction. Two days post transduction, successful transduction was detected by X-Gal staining. Vector titers from one representative experiment are shown.

The evaluated vector titer was 6 x 10^4 i.u. and thus hardly as high as the vector amount employed in these experiments, indicating that almost all pseudotyped vector particles were able to infect the permissive T-cell sub-population among the PBMCs. Using higher multiplicities of infection (M.O.I. = infectious vector particle to target cell ratio), we were not able to achieve higher transduction efficiencies (data not shown), suggesting that only about 10% of the PBMC were susceptible to the respective MLV(HIV-1) pseudotype vectors. We further hypothezised that the

observed enhanced titers on αSDF-1β-treated PBMCs reflected the up-regulation of the SDF-receptor CXCR4 induced by the decreased concentration of SDF in the culture medium. CXCR4 was shown to function as a co-receptor for the MLV(HIV-1) vector particles and is needed to allow efficient cell entry (Fig. 2). Therefore, the expression-level of CXCR4 may have an impact on the efficiency of gene transfer mediated by the respective particles.

DISCUSSION

We have previously shown that an *env* gene variant encoding a C-terminally truncated transmembrane protein and a full-length surface protein of HIV-1 was instrumental to generate MLV(HIV-1) pseudotype vector particles upon its expression in otherwise *env*-negative MLV-derived packaging cells (Schnierle & Stitz *et al.*, 1997). Previously described packaging cell lines produced only moderate vector titers of up to 9×10^4 i.u./ml. We report here the generation of new packaging cell lines that allow the harvest of supernatants containing more than 1×10^6 i.u./ml of respective vector particles. The efficiency of vector production is thus comparable with that of stable amphotropic packaging cell lines (Markowitz *et al.*, 1988; Cosset *et al.*, 1995) used in today's clinical gene therapy trials. The described MLV(HIV-1) vector particles were shown to allow simple concentration employing ultrafiltration devices. Using this method, vector stocks with titers of up to 2×10^8 i.u./ml could be generated that should be considered sufficient for possible future clinical trials. We also demonstrated the successful transduction of primary human T-cells. However, transduction seemed to be limited to a small subpopulation of the PBMC and could not be increased using higher vector doses. It is conceivable to conclude from these data that only a small portion of the primary T-cells used here expressed the CD4 receptor in conjunction with the CXCR4 co-receptor and was in a state of active proliferation. This is in

agreement with reports showing that only a very small subpopulation of human T-cells can be infected with HIV (Bruunsgaard *et al.*, 1995; Zhang *et al.*, 1998). The MLV(HIV-1) vector particles described here were shown to specifically transduce human CD4/CXCR4-positive cells which are the natural host cells for T-cell tropic HIV-1 strains. Thus, MLV(HIV-1) vectors may be valuable tools to deliver anti-retroviral genes into the host cell compartment of HIV-1. Anti-retroviral therapeutic genes delivered by respective pseudotype vector particles could for example encode ribozymes, transdominant-negative mutant HIV proteins, RNA-decoys or antibody-fragments directed against HIV-1 proteins (reviewed in: Yu *et al.*, 1994; Pomerantz *et al.*, 1995). This would lead to intracellular immunisation of the natural host cells against infection by HIV. Unfortunately, the use of the MLV(HIV-1) vector particles for *in vivo* applications would probably be restricted to HIV-1 seronegative individuals. The humoral anti-HIV-1-immune response of HIV-1-infected patients would presumably lead to the neutralisation of the MLV(HIV-1) vector particles *in vivo* before transduction could occur. In contrast, the presence of HIV-1 derived envelope proteins in the respective vectors could be of benefit when using them as vaccines to induce immunity in healthy recipients. These vector particles would induce a humoral immune response against HIV-1 Env and are thus potentially beneficial in uninfected individuals.

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